THE AUSTRALIAN JOURNAL OF EXPERIMENTAL BIOLOGY AND MEDICAL SCIENCE

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VOL. IV

PUBLISHED BY THE UNIVERSITY OF ADELAIDE
MANAGED FOR THE UNIVERSITY BY THE MEDICAL SCIENCES CLUB OF SOUTH AUSTRALIA

PRINTED AT THE HASSELL PRESS, ADELAIDE
1927

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ON SOME CONDITIONS AFFECTING THE VIABILITY OF CULTURES OF INFUSORIA AND THE OCCURRENCE OF ALLELOCATALYSIS THEREIN

by

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(Submitted for publication 11th December, 1926.)

1. *Conditions affecting the occurrence of Allelocatalysis.*

In the course of investigations carried out during the past several years upon the conditions affecting the reproduction of Infusoria in hay infusion, I found that the rate of reproduction is affected in two different ways by the density of the infusorial populations. In young cultures it was found that reproductive rate increased with density of population, while in old, thickly inhabited cultures the density of population appeared on the contrary to be an inhibiting factor, for reproduction ceases, although the culture fluid, after removal of the infusoria, is still able to support the multiplication of infusoria which are freshly inoculated into it (14).

The accelerative effect of increasing population in young cultures is evidenced in diverse ways. Thus the rate of production of new individuals in subcultures rises in an autocatalytic curve as the population increases. It was further found, moreover, that single infusoria, isolated into small volumes of fresh culture medium, reproduce more rapidly than infusoria from the same parent culture which are isolated into larger volumes of culture medium (15). This observation Cutler and Crump have been unable to confirm (4). Again, it was found that two individuals isolated together into the same small volume of culture medium reproduce more rapidly than a single individual isolated into a like volume of the same medium (allelocatalytic effect). This observation also Cutler and Crump have failed to confirm, while Peskett (10) has been unable to obtain any evidence of the occurrence of the phenomenon in cultures of yeast. Finally, I found that allelocatalytic effect is augmented and the reproductive rate diminished by washing the infusoria prior to their introduction into the culture (17, 18); these observations, furthermore, Cutler and Crump (6) and Peskett (10) have not been able to repeat.

Before entering upon a discussion of some possible origins of these discrepancies it is necessary to draw attention to the fact that the phenomenon of allelocatalysis is by no means confined to cultures of infusoria, but, on the contrary, appears to be of very general occurrence. Thus it has been shown by Burnet (1) to occur in cultures of bacteria. Fischer (7) has found that in connective-tissue cultures *in vitro* single fibroblasts do not multiply, growth taking place only when the cells are numerous and close to one another. Burrows and Suzuki have noted analogous phenomena (2). The occurrence of more than one autocatalytic cycle of growth in vertebrate animals shows that certain cells in the animal share a common catalyzor, while others, which are not influenced by this catalyzor, produce a growth-catalyzor of their own, which they share among themselves, with the result that their growth gives rise to a separate autocatalytic cycle of weight-increment (19). The effect of *failure* to share a common catalyzor in thus rendering the processes of growth independent of one another, shows that, when growth involving a multiplicity of cells and tissues yields weight-increment which is defined by a single curve of autocatalysis, all of the cells concerned must be affected by the growth of each of them, or in other words, that some common catalyzor is diffused throughout the whole mass, being produced by every cell in the group or community, and affecting the growth of each of them in a like manner. The growth of a bird or mammal consists of two or three mutually independent autocatalytic cycles, because these animals contain two or three such communities of cells, which differ from one another in the nature of the catalyzor which they share. Allelocatalysis, therefore, must occur in the growth of those communities of cells which comprise the higher animals and plants, since the growth of all of these which have been investigated seems to consist of one or more autocatalytic processes.

If, therefore, communities of yeast or infusoria should chance not to display allelocatalysis, their behaviour would constitute an exception to the usual behaviour of cell communities, and it would become of some importance to ascertain the origins of this departure from the general rule.

Before proceeding to describe the investigations which form the second part of this paper, it is necessary to dwell upon certain differences between the technique employed by Cutler and Crump and that which I have employed, since the disagreement of our results may originate in any of these, or in several of them combined.

In the first place, attention must once more be drawn to the fact that until the experiments were performed which are described in their most recent paper (6), Cutler and Crump did not employ washed individuals in their experiments upon allelocatalysis, whereas I have always washed the isolated individuals at least once (unless the contrary is specifically stated), and usually twice, in relatively large volumes of fresh culture medium before employing them to test for the mutually accelerative effect of pairs of individuals upon each other's

reproduction. Cutler and Crump have endeavoured to insinuate that this was not the case, by quoting my words describing an experiment in which it is not specifically stated that the individuals employed were washed. Because it was not stated to have been done in this individual experiment it is not to be inferred that the washing was omitted, and, in fact, this precaution appeared to me, from the very first experiment of this nature which I performed, to be so very obviously a necessary one that it never occurred to me to omit it, except with the deliberate intent of ascertaining the effect of *failure* to wash the animals before subculturing them. Presumably, if contiguous animals can affect each other's growth without the occurrence of conjugation, they must do so through the agency of some soluble substance which they emit, and which is transferred from one organism to the other through the medium which they inhabit. Presumably, therefore, also, this soluble substance must be very abundant in the thickly inhabited cultures from which subcultures are usually prepared. Therefore the removal of adherent parent-culture medium from the bodies of the individuals employed for inoculation was an obviously necessary preliminary to the discovery of allelocatalytic effect, if any such effect occurred. Later investigations showed, in fact, that washing of the infusoria leads not only to removal of adherent parent-culture fluid, but also to washing out of accumulations of autocatalyst from the protoplasm of the cells themselves (17).[1]

Another difference between the techniques employed by Cutler and Crump and myself resides in the fact that instead of employing the "natural" medium, to wit, hay infusion, in which my observations have been made, they employ a "synthetic medium," originally devised by Peters to show that reproduction of protozoa could take place in an artificial medium and in the absence of associated bacteria (12). Instead of utilizing this discovery of Peters, however, and cultivating their infusoria in bacteria-free media, they introduce bacteria (Sarcina) into the medium as well, thus introducing the very complication (that of simultaneous growth of two organisms in the same medium) which the medium appeared to have been designed to avoid.

The results obtained by Peters, in fact, have failed to receive confirmation, and the observations of Cutler and Crump themselves indicate that the complication arising out of the growth of associated organisms is an unavoidable one, because non-parasitic infusoria will not grow in their absence. Thus Cutler and Crump have shown that the culture of *Colpidium* employed by Peters was contaminated by an unidentified species of small bacillus; that when these are scanty the ciliates show "typical signs of hunger degeneration," and that when they are abundant the growth of the *Colpidia* is greatly stimulated (5). Furthermore, it has been shown by Cutler and Crump that the multiplicative rate of *Colpidia* increases with the ratio of these bacteria to the ciliates inhabiting the medium.

[1]In my first article on this subject it was shown that washing with distilled water prevents allelocatalytic effect. This, however, is entirely distinct from the effect of washing the infusoria with the medium into which they are subsequently subcultured.

Two possibilities now suggest themselves: If, on the one hand, the ciliates actually feed only upon these "contaminating" bacteria, then their growth must obviously determine the growth of the infusoria and the density of the original bacterial inoculum will determine the initial rate of growth of a subculture, not the number of infusoria. Thus two infusoria inoculated together into a given volume of culture medium may be prevented from multiplying more rapidly than a single individual, simply because the food-supply is inadequate to permit reproduction beyond a certain maximum rate, no matter what stimulating substances or catalyzors may be present. In all cultures, then, containing one, two, three, or more infusoria in the original inoculation, we may merely be obtaining a growth rate which is rendered uniform by the uniform magnitude of the original bacterial inoculation, or else a growth-rate in which the diminished bacteria-ciliate ratio counterbalances or overshadows the allelocatalytic effect due to the presence of more than one ciliate in the subculture.

If, on the other hand, the infusoria can live upon Sarcina as well as upon the "contaminating" bacillus, then the further question arises as to the constancy or otherwise of the bacteria-ciliate ratio in cultures containing varying initial numbers of infusoria. For if this ratio has the importance which Cutler and Crump's results indicate, then the introduction of equal numbers of Sarcina into each of the cultures containing one, two, or three infusoria would lead to diminished reproductive rate in the cultures containing the greater population of ciliates, and allelocatalytic effect would tend to be counterbalanced by paucity of food supply. So far as one can judge from their description of their technique (which is somewhat indefinite in respect to this point), it appears that Cutler and Crump introduced equal numbers of Sarcina into each culture. The counterbalancing effect of diminishing bacteria-ciliate ratio may therefore have actually contributed in their experiments to mask allelocatalytic effect.

In my own experiments the bacterial inoculum must be very nearly proportional to the number of ciliates introduced, for since the infusoria were washed prior to inoculation into the culture medium, the bacteria introduced with them must have been chiefly those which were closely adherent to the bodies of the ciliates or engulfed within their vacuoles. The bacteria-ciliate ratio must therefore be nearly constant. This raises once more the question, however, whether the allelocatalysis which I have observed is really due to the mutually accelerative action of the ciliates themselves, or to an underlying allelocatalysis of associated bacteria. This possibility occurred to me immediately after obtaining my first positive results, and I repeated my experiments on allelocatalysis, employing media heavily infected with bacteria by exposure to air for 24 hours. I found that allelocatalysis occurred in these "bacterized" infusions no less than in fresh hay infusion. Cutler and Crump, however, have shown that the growth of *Colpidium* is very much more facilitated by the "contaminating bacillus" which was found by them to be associated with this organism than it is by the Sarcina

which they employed as a food-organism. This suggests the possibility that *Colpidia* and other ciliates can live only upon particular species of bacteria, and that other bacteria which may be present only affect their growth to the extent that they modify the nutrients available for the food-species of bacteria. If this be the case, then my experiment, upon which I relied to show that allelocatalysis originated in the contiguity of the ciliates and not in that of the associated bacteria, is inconclusive, because the bacteria which develop spontaneously in hay infusion may not be those which the ciliates can employ as food, and the question of the origin of allelocatalytic effect in infusorial cultures still remains unsettled.

Whether the allelocatalytic effect originates with the ciliates, or with associated food-organisms, however, it remains a mutually accelerative effect of contiguous organisms upon their reproductive rates. That is, the contaminating organisms multiply much more than twice as rapidly when the mass of the inoculum is doubled. In fact, if the effect really originates with an associated "contaminating organism," then the estimate of its magnitude which is afforded by the reproductive rates of the infusoria is very inadequate, for, according to the figures supplied by Cutler and Crump, the reproductive rate of the infusoria increases much more slowly than the population density of the food organisms. Nor, if this should eventually be ascertained to be the origin of the allelocatalytic effect which I have observed, does it at all preclude the possibility that allelocatalytic effect also occurs between contiguous pairs of infusoria. To demonstrate its existence, however, would necessitate inoculating the infusoria into a culture-medium already densely inhabited by the food-organism, so that the additional organisms introduced with the ciliates would form an inappreciable proportion of the number already present, but as I am ignorant, at present, of the nature of the food-organisms required by the species of ciliates which I employ, I have been unable to perform this experiment.

If, however, the growth-rate of infusoria is determined by the multiplication of food-organisms which are associated with them, then it is curious that Cutler and Crump should have failed to ascertain any effect of washing the ciliates upon their reproductive rate, more especially since they have suggested that the low maximal populations of ciliates obtained by Peters were due to the absence of any but minimal populations of the contaminating food-organism. It is also peculiar that they have failed to find any favourable influence upon the reproductive-rate of the ciliates, of reducing the volume of the culture-medium into which the inoculum is introduced. Both of these effects would appear to be indicated by their own results. Their failure to obtain such effects suggests that *Colpidium* can utilize *Sarcina* as a source of food as well as the "contaminating organism," and that the allelocatalysis of the associated food-organisms, and the above-mentioned effects as well, were masked by the excess of food-organisms initially introduced into the culture with the added emulsion of *Sarcina*.

Finally, in dwelling upon this possibility, it must be stated that all of the data and conclusions concerning allelocatalysis in infusoria which have issued from this laboratory in recent years remain valid, save that, if the above interpretation is correct, they apply to the associated food-organism and not to the infusoria themselves.

The differences of reproductive-rate among different subcultures of infusoria is extremely great, varying with the age, population, and previous history of the parent-culture, as well as with temperature and the character and volume of the culture-medium employed. For this reason, in the study of allelocatalytic effect, I have always compared the reproductive rates of single individuals and of pairs of individuals, respectively, which are derived from the same parent-culture. Provided care is taken not to consume too much time in abstracting the individuals for isolation (cf. part 3 of this article), the reproductive rates of different individuals isolated from the same parent-culture are almost always identical. Cutler and Crump, however, compare and average the reproductive rates of different cultures containing one, two, or three individuals respectively, which are derived from different parent-cultures of the same age. This leads, naturally, to a great range of variability among the reproductive rates obtained. I pointed out in a previous article, that their (unwashed) single-cell cultures displayed a progressively *diminishing* reproductive rate with diminishing volume of the culture-fluid, suggesting the presence of some inhibiting substance derived from the parent-culture medium. Cutler and Crump maintained, in reply, that the variations of reproductive rate lay within the normal range of variability, and that their progressive augmentation in the order of increasing volumes of culture medium was accidental. In my experience, employing the technique which I have described, they greatly exceed the normal range of variation of reproductive rates in subcultures (derived, that is, from a single parent-culture). Obviously, in seeking for the presence or absence of substances affecting reproductive rate, it would be advisable to employ cultures which, in the absence of the factors sought, might be relied upon to display uniform reproductive rates.

Attention must also be drawn to the fact that allelocatalysis is displayed only between pairs of *living* cells. I have repeatedly investigated the effect of dead infusoria upon the reproduction of living infusoria introduced into the medium containing them. In the volume of medium usually employed for subcultures (0·08 to 0·10 cc.) a single dead *Enchelys* (killed by heating to 40°C) has no effect whatever upon the multiplication of a living individual inoculated into the medium. A second *living* individual, on the contrary (provided that the infusoria have been washed prior to their inoculation into the medium) may accelerate the reproductive rate of each of them to such an extent as to quadruple or even multiply sixteen times the population of the culture at the end of 24 hours Robertson, 14, 16).

It follows from this that if the conditions of inoculation or the character of the medium are such as to lead to an extensive mortality among the individuals which form the subcultures, allelocatalytic effect must be obscured, and that if the mortality attains to 50% of the individuals isolated, allelocatalytic effect must fail altogether to be displayed, because, in the average culture, one of the two individuals inoculated together must die, and the final effect must be the same as that obtained in cultures of single individuals which chance to survive. Unless the cultures were continuously watched in order to observe the multiplication of each of the individuals isolated, comparison of the end-results, coupled with rejection of those cultures which failed to reproduce at all, would obviously lead to the conclusion that allelocatalytic effect does not occur.

So far as the failure of Peskett to observe allelocatalysis in cultures of yeast is concerned, this is all the comment that I propose to make, in view of his admission that the mortality in his cultures ranged from 28% to 50% (10). Whether allelocatalysis occurs in cultures of yeast or not, I cannot say, for I have no experience in the culture of yeasts, but it is perfectly evident that allelocatalysis can never be demonstrated in cultures of any cells among which the mortality may attain 50%. Even one-half of this rate of mortality must obviously mask the effect to a very considerable extent.

It is possible that this factor has also played an important part in masking allelocatalytic effect in Cutler and Crump's experiments upon reproductive rate in *Colpidium*, for they have admitted that in the media which they employ a considerable mortality is common among subcultured individuals (3), and they were careful to point out in one of their earlier papers (4) that "where only one animal is present any growth must be due to that animal, but where two are introduced there is always the possibility that one may have died," but in their recent paper on this subject (6) this possibility is not recalled.

Furthermore, death may occur in these media, not only among the animals originally inoculated into the medium, but also, as Cutler and Crump have themselves shown, among the animals which are produced in the subculture itself, so that the numbers of living animals present after 24 or 48 hours may afford a very misleading estimate of the number actually produced. In my own experiments I have also, in certain media and under certain conditions, observed deaths among the inoculated individuals, and, as will be shown below, diminution in the population of fairly heavily populated cultures. But experiments upon allelocatalysis have never been undertaken excepting under conditions permitting reproduction of all of the subcultured individuals, a fact which was established by hourly examination of the cultures. The origin of the discrepancy between my results and those of Cutler and Crump may therefore be found to depend ultimately upon the discovery of the origin of the deficient and fluctuating viability of cultures in the artificial media employed by these authors, and it was with this object in view that the investigations were undertaken which are about to be described.

2. *Factors influencing the viability of cultures of Infusoria.*

One of the most important factors in determining the viability of subcultures of *Enchelys* is, as stated in earlier papers, the temperature at which the culture is maintained. Subcultures into hay infusion, adjusted to an optimum P_H, almost always die at temperatures above 30°C. The more numerous the inoculum the more chance there is of survival and multiplication of some of the individuals forming the subculture, but the numbers always decrease for some time after subculturing. During the same period the parent-culture remains maximally populated, so that density of infusorial population forms in itself a protecting agent against the adverse effect of high temperature.

The laboratory temperature in South Australia not infrequently rises above 30°C. during the summer, and since it was found impossible to obtain uniform reproductive rates in subcultures in the neighbourhood of this temperature, work upon allelocatalysis has always been discontinued in this laboratory during the summer months. The cultures were maintained, however, by mass-inoculation at frequent intervals, and various endeavours have been made to ascertain the cause of the high mortality in cultures at these temperatures as well as means of overcoming it.

In the course of these investigations two means were found of ensuring the viability of subcultures, provided that the temperature at which they were maintained did not too greatly exceed the limit of 30°C. One consisted in pre-cooling the parent-culture to 4°C. for 24 hours preceding the abstraction of individuals to form the subculture. The age of the parent-culture has a great influence upon the viability of subcultures. Subcultures from very old parent-cultures frequently die, even at temperatures of 20°C. or less. At higher temperatures the infusoria multiply more rapidly, so that the effective physiological age of the cultures at a given number of days is greater at a higher than it would be at a lower temperature. It would seem possible that the cooling of the parent-culture, by slowing or arresting multiplication, practically maintains its physiological age at that of the preceding day. That this is not the sole cause of the greater viability of subcultures from pre-cooled parent-cultures is shown by the fact that these cultures may frequently survive when subcultures made the preceding day from the same parent-culture uniformly failed to survive.

Another way of promoting the viability of subcultures proved to be that of allowing the culture fluid to stand, at room temperature, for 24 hours before subculturing into it. As stated in a former paper, the reproductive rates of infusoria inoculated into this "bacterized" culture fluid are always greater than the reproductive rates of infusoria inoculated from the same parent-culture into unbacterized culture fluid. Not only is reproductive rate increased, however, but also viability. In warm weather it became our custom, when subculturing, always to keep the unused excess of culture fluid until the following day. If

the subcultures had failed, then fresh subcultures into the same culture fluid 24 hours after its preparation almost invariably succeeded. That this is due to an alteration of the culture fluid and not of the parent-culture during the interim is shown by the fact that if subcultures are simultaneously inoculated from the same parent-culture into freshly prepared culture medium and medium which is 24 hours old, the latter survive, while the former do not. On the other hand, culture fluid incubated at higher temperatures (37·5°C.) for 24 hours is toxic, and subcultures will not survive in it.

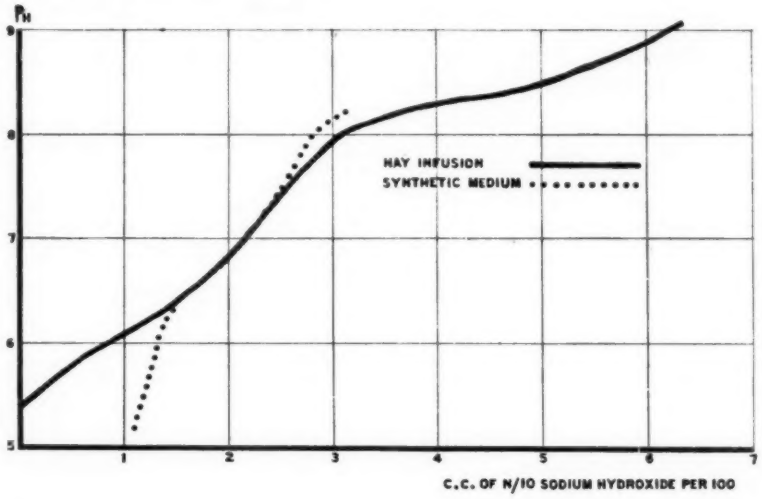

Figure 1. Showing the effect of added acid or alkali upon the P_H of hay infusion and that of synthetic culture medium. To facilitate comparison the titration-curve of the synthetic medium has been shifted so that the two curves coincide at neutrality ($P_H = 7·20$). Reactions more acid than this, in the case of synthetic medium, were actually procured by addition of acid equivalent to the alkali indicated on the base-line.

During the course of experiments on the effect of various dyes upon the reproduction of infusoria, it was found that densely populated cultures of *Enchelys* became alkaline, since phenolphthaleon added to the culture fluid, in amounts insufficient to be toxic, turned red as the cultures became heavily populated, indicating a P_H of at least 8·3. Since this alkalinity (also observed by Cutler and Crump) (3) is probably attributable to the metabolic activities of the bacteria contained in the cultures, it appeared possible that the favourable effect of allowing the culture fluid to stand for 24 hours before employing it might arise from an alteration of its P_H. Accordingly, it became of importance to determine the changes of P_H which infusorial cultures undergo with age.

As a preliminary to this investigation freshly prepared hay infusion,

obtained by heating 5 grms. of chopped oaten hay in 100 cc. of distilled water until it just begins to boil, transferring to a boiling water bath for one hour, filtering, and making up the volume of the filtrate, if necessary, to 75 cc., and finally aerating with a stream of filtered air for one hour, was titrated against acid and alkali, and the resultant P_H values were determined electrometrically. I am indebted to Mr. Hedley R. Marston, of this department, for carrying out these titrations, the results of which are graphically displayed in figure 1.

It is at once evident from inspection of these graphs that the synthetic medium is much less efficiently buffered, both on the alkaline and on the acid side, but especially on the latter, than the hay infusion. It is interesting to note that hay infusion is especially well buffered in the neighbourhood of $P_H = 8$ to 8·5, which is the stable final condition of a mature culture of *Enchelys*.

TABLE 1.

Hay Infusion.		Synthetic Medium.[2]	
C.cm. of Alkali added per 100 cc. of Medium.	P_H of Mixture.	C.cm. of Acid or Alkali added per 100 cc. of Medium.	P_H of Mixture.
0·00 cc. $\frac{N}{10}$ NaOH	5·41	1·20 cc. $\frac{N}{10}$ HCl.	5·17
0·44 „ „	5·75	1·00 „ „	5·92
0·90 „ „	6·04	0·80 „ „	6·35
1·36 „ „	6·32	0·60 „ „	6·56
1·81 „ „	6·66	0·40 „ „	6·78
2·26 „ „	7·15	0·24 „ „	6·93
2·71 „ „	7·66	0·08 „ „	7·12
3·16 „ „	8·05	No addition	7·22
3·62 „ „	8·22	0·16 cc. $\frac{N}{10}$ NaOH	7·30
4·07 „ „	8·30	0·32 „ „	7·69
4·52 „ „	8·39	0·48 „ „	7·98
4·97 „ „	8·52	0·80 „ „	8·20
5·42 „ „	8·66		
5·90 „ „	8·88		
6·33 „ „	9·08		

[2]The composition of this medium was as follows (Cutler and Crump, 3):

Na_2HPO_4	0·001%	$MgSO_4, 7H_2O$	0·0001%
Ammonium Lactate ..	0·01%	$CaCl_2$	0·002%
KCl.	0·03%	Glucose	0·04%
NH_4Cl.	0·03%		

The initial reaction of the mixture largely depends upon that of the ammonium lactate. In preparing the medium for the above determinations the ammonium lactate was introduced by mixing standardized solutions of lactic acid and ammonia in the proportions found necessary to secure a reaction of the mixture corresponding to $P_H = 7·2$.

These data, which are enumerated in Table 1, enable us to compute the initial P_H of hay infusion to which definite proportions of alkali have been added. In order to ascertain the effect of initial P_H upon the viability, duration of lag, and reproductive rates of subcultures, 10 cc. samples of aerated hay infusion were mixed with 0·1, 0·2, 0·3, 0·4, and 0·5 cc of $\frac{N}{10}$ NaOH solution respectively, corresponding to initial P_H's of 6·1, 6·8, 7·9, 8·3, and 8·5. Subcultures were prepared in each of these media from the following parent-cultures: 182A (seven days old), 183A (three days old, kept for the past 24 hours at 4°C.), and 185A (three days old, kept at room temperature throughout). The room temperature was 22°C. A small counted number of individuals was inoculated into each subculture, and no attempt was made to wash them, since all that it was desired to ascertain was the presence or absence of toxicity of the media. The following were the results obtained:

TABLE 2.

Subculture. Number.	Parent-culture.	P_H of Medium.	Initial No. in. Culture.	No. Inhabiting Subcultures after 18 hrs.	19 hrs.	20 hrs.	21 hrs.	24 hrs.	42 hrs.
197A	7 days old	6·1	9	1	2	2	2	4	73
198A	„	6·8	8	6	6	7	11	17	358
199A	„	7·9	9	4	4	4	4	8	250
200A	„	8·3	10	11	13	15	16	25	502
201A	„	8·5	10	4	4	4	8	14	231
202A	3 days old, pre-cooled	6·1	7	14	—	—	18	33	—
203A	„ „	6·8	13	41	—	—	76	138	—
204A	„ „	7·9	9	43	—	—	67	91	—
205A	„ „	8·3	9	40	—	—	88	139	—
206A	„ „	8·5	9	39	—	—	67	121	—
207A	3 days old	6·1	14	21	—	—	39	58	—
208A	„	6·8	12	52	—	—	95	163	—
209A	„	7·9	10	38	—	—	56	112	—
210A	„	8·3	9	34	—	—	56	113	—
211A	„	8·5	9	31	—	—	52	95	—

It is evident that the duration of lag, which in subcultures from the seven-day culture lay between 18 and 20 hours, is not appreciably affected by P_H within the range employed. Viability in the most acid culture ($P_H = 6·1$) is diminished, and perhaps for this reason the 24 hours reproductive rate is also diminished. We may infer that an initial P_H in the neighbourhood of 6·1 is definitely deleterious to the organisms, and that in cultures from old parent-cultures the optimum initial P_H is in the neighbourhood of 8·3, the final P_H.

that is, of an adult culture. Subcultures from younger parent-cultures, however, maintain an approximately uniform reproductive rate in subcultures in which the initial P_H varies between 6·8 and 8·5.

Having thus established the lower limit of toleration of *Enchelys* for variation of initial P_H in the medium, an experiment was undertaken to ascertain the variation of P_H and bacterial and infusorial populations of cultures during the process of multiplication. The estimations of P_H were carried out by Mr. Hedley R. Marston, and the bacterial counts by Mr. F. Draper, to both of whom I am greatly indebted for their assistance.

The parent-culture was a mass-culture 500 cc. in volume, which had originally been inoculated with 40 cc. of a thickly inhabited culture, and was 18 days old. This was spread in a layer about 0·5 cm. thick over an area of about 1,000 sq. cm., forming the bottom of a glass bowl 18 cm. deep, 26 cm. in diameter at the bottom and 36 cm. at the top. This was covered by a glass electric light reflector, which rested upon the edge of the bowl, and was fastened to the bowl by strips of adhesive plaster. The hole at the top of the reflector was plugged with cotton wool. The subcultures were kept in similar containers, and samples were withdrawn from time to time for the determination of P_H and bacterial count through the opening in the top of the reflector.

Although the parent-culture was 18 days old, and the temperature of the laboratory varied between 22·5° and 23·5° C., the population, estimated from the population of diluted samples, was only 100 per 0·1 cc., and contained many dividing individuals. This difference between the densities of population attained in drop-cultures and those attained in like time in mass-cultures was invariably observed. From this it is evident that cessation of reproduction in cultures is not due to age, in itself, or to bacterial multiplication, or to the variation of P_H induced by bacteria, but directly to the previous multiplication of the infusoria themselves. This, occurring as it does but slowly in mass-cultures, continues to occur, in consequence, for a prolonged period. The probability is that in layers 0·5 cm. deep oxygen is the limiting factor in determining rate of development. Multiplication would therefore occur only in the surface layers, while the accelerative (and ultimately retarding) substances produced during division are distributed through the whole mass of the fluid by diffusion, so that the concentration of autocatalyst in the region to which multiplication is confined does not correspond to the extent of multiplication which has occurred, and the cultures therefore age more slowly than drop-cultures in which oxygen is available in excess.

A litre of hay infusion was prepared by heating 67 grms. of chopped oaten hay with 1,333 cc. of distilled water to boiling, and then heating on a boiling water bath for one hour. The mixture was filtered, and the filtrate, when cool, made up to 1,000 cc. with distilled water, and aerated with a strong current of air (filtered through sterile wool) for one hour. The aerated filtrate was divided

into two portions of 500 cc. each. One was buffered with 20 cc. of $\frac{N}{10}$ $NaHCO_3$ and the other with 18 cc. of $\frac{N}{10}$ NaOH. The culture fluids were then placed in receptacles as described above, and each of them was inoculated with 20 cc. of parent-culture (without washing). Immediately after inoculation and thorough mixing, three $\frac{1}{10}$ cc. samples were withdrawn from each subculture and placed in the central depressions of sterile slides, which were kept upon pads of wet filter paper in Petri dishes for observation of the increase in infusorial population. At the same time, and at intervals subsequently, larger samples were withdrawn for the determination of P_H and bacterial count. The P_H values were determined electrometrically in a Clarke shaking electrode, the bacterial counts by dilution and spreading measured volumes over the surfaces of sterile agar plates, which were subsequently incubated, and the colonies counted.

The following were the results obtained:

TABLE 3.

Time (Hours)	Infusoria per Individual originally introduced (Average 3 per 0·1 cc.) NaOH Culture.	Infusoria per Individual originally introduced (Average 3 per 0·1 cc.) $NaHCO_3$ Culture.	P_H NaOH Culture.	P_H $NaHCO_3$ Culture.	Bacteria per cc. NaOH Culture.	Bacteria per cc. $NaHCO_3$ Culture.
0	1	1	8·65	7·30	$0·50 \times 10^6$	$0·43 \times 10^6$
2·5	1	1	8·45	7·50	$2·00 \times 10^6$	$1·89 \times 10^6$
19·0	10·7	11·0	7·15	7·66	$47·70 \times 10^6$	$49·00 \times 10^6$
26·5	22·9	17·0	6·93	7·34	$86·60 \times 10^6$	$108·50 \times 10^6$
43·0	23·5	12·3	7·20	7·81	$3·30 \times 10^9$	$3·80 \times 10^9$
67·0	200 (about)	152 (1 culture only)	7·98	7·98	$48·00 \times 10^9$	$52·80 \times 10^9$
91·0	—	—	8·10	8·10	$9·98 \times 10^{12}$	$10·78 \times 10^{12}$
115·0	—	—	—	—	$101·0 \times 10^{16}$	$123·0 \times 10^{16}$
139·0	—	—	—	—	$66·0 \times 10^{18}$	$83·0 \times 10^{18}$

It is evident that the bacterial counts in both cultures were the same within the experimental error. A scum formed upon the surface in both cultures on and after the third day. As this presumably represented bacteria and bacterial products not available for the nutrition of the infusoria, the samples withdrawn for counts were taken from below the scum, but, on account of the scum, the cultures could not be mixed before taking the samples.

Despite the similarity in bacterial contents, however, the rate of infusorial reproduction differed considerably in the two cultures. The production of infusoria in both cultures was checked during the second 24 hours, but in the culture buffered with $NaHCO_3$ the number of infusoria actually underwent diminution, so that at 43 hours the infusorial population in the $NaHCO_3$ culture

was about half that in the NaOH culture. In the succeeding 24 hours the infusorial population underwent great increase in both cultures. These observations correspond to those reported by Cutler and Crump (3, 5), who observed irregular fluctuations of reproductive rate in their cultures of *Colpidium*,

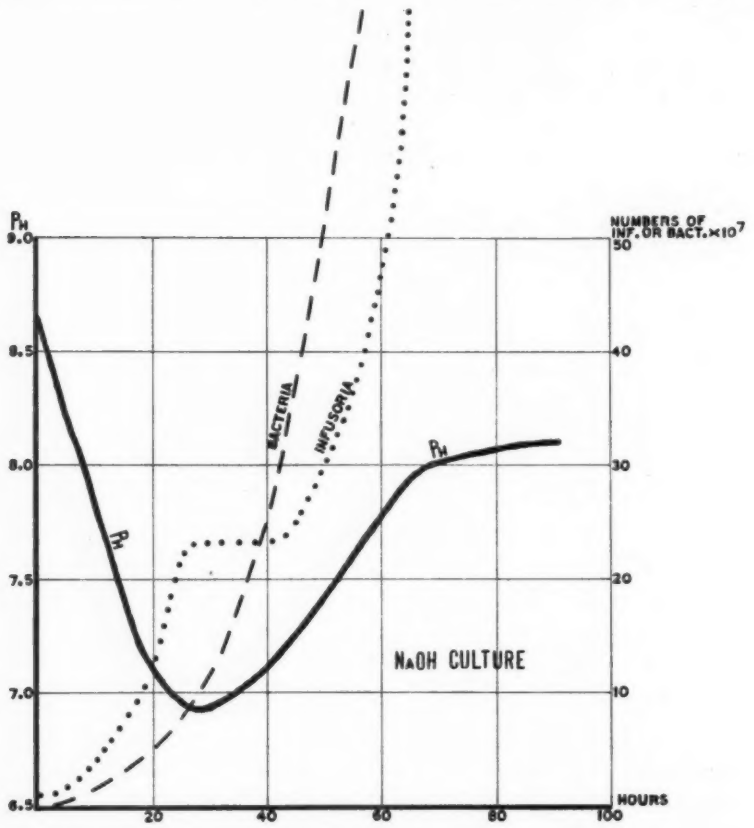

Figure 2. Showing the changes of P_H and bacterial and infusorial populations subsequently to inoculation of hay infusion originally brought to $P_H = 8.65$ by addition of NaOH. Temperature 22.5°C. to 23.5°C.

and frequently temporary diminutions of population. It would appear from the above-cited results that these fluctuations do not originate in fluctuations of bacterial population. It must be admitted, however, that the evidence of this is inconclusive, because, in the first place, we are not certain that the type of bacterial population, which is estimated by growth on agar plates, is the type

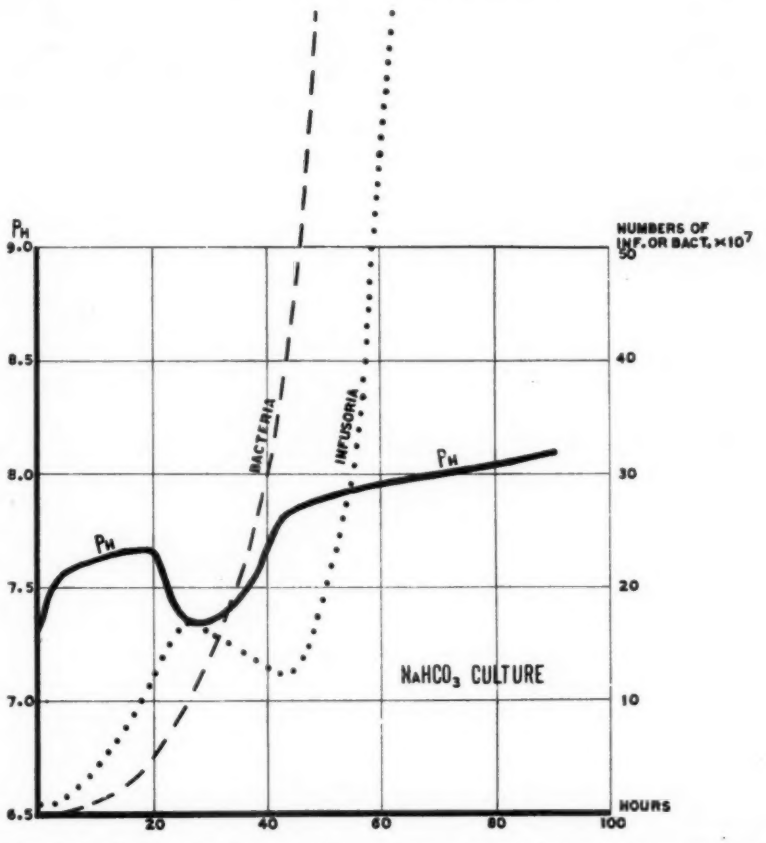

Figure 3. Showing the changes of P_H and bacterial and infusorial populations subsequently to inoculation of hay infusion originally brought to $P_H = 7.30$ by addition of $NaHCO_3$. Temperature 22.5°C. to 23.5°C.

upon which the multiplication of *Enchelys* depends, and in the second place, the infusorial population was estimated in drop-cultures, while the bacterial population was estimated in bulk-cultures.[3]

On the other hand, the estimates of the P_H values show that in both cultures the P_H attained a minimum value at 24 hours, coincidently with the check in reproduction of the infusoria (cf. figures 2 and 3). It is curious, however, that

[3]The infusorial population could not be estimated directly in the bulk-cultures, because the presence of the bacterial scum after the second day prevented mixing of the cultures, and the infusoria were not distributed evenly through them, but, as explained above, were concentrated in the surface layers.

the most severe check upon reproduction of the infusoria occurred in the $NaHCO_3$ culture in which the minimum P_H was 7·34, while in the NaOH culture, which suffered less, the P_H fell as low as 6·93. As 16½ hours elapsed before the next P_H determination was made, we cannot be sure that the observed minimum of P_H was the actual minimum, but it is evident that in the $NaHCO_3$ culture in 7½ hours, during which the P_H fell from 7·66 to 7·34, a definite check had been experienced by the infusoria, the effect of which endured for a considerable time, while a fall from 7·15 to 6·93 in the NaOH culture during the same period was less injurious. It may be that the P_H changes in the bulk-cultures did not faithfully reproduce the changes occurring in the drop-cultures, which may have been more rapid or more profound, but, on the other hand, it is possible that in the $NaHCO_3$ culture the CO_2 tension was higher, so that the effect of outside P_H upon the P_H in the cells themselves was greater than it was in the NaOH culture, since the penetrability of cells for CO_2 is greater than their penetrability for other acid radicles (Jacobs, 1920).

Whatever its origin, however, it is evident that in these cultures, whether initially alkaline (8·65) or neutral (7·30), the P_H diminishes either immediately (in the initially alkaline culture) or after a period of increase (in the initially neutral culture), so that in either case there is a phase of relative acidity which culminates at about 24 hours, under the conditions of temperature, etc., which are employed in this experiment. Subsequently to this the P_H increases until it attains a value lying between 8·0 and 8·3, which is the final condition attained in both the cultures, irrespective of the value of the initial P_H. It is in this region also that hay infusion is most effectively buffered (figure 1).

During the phase of relative acidity the conditions are injurious for the infusoria. Obviously, if the culture medium is inadequately buffered in the region between $P_H = 6·0$ and 7·0, the chance of arrest of multiplication, or even death, of the infusoria must be much greater than in media which are more effectively buffered in this region. On the acid side of $P_H = 6·4$, and on the alkaline side of $P_H = 7·8$, the synthetic medium employed by Cutler and Crump is much less effectively buffered than hay infusion. It is therefore a less efficient medium, both for preserving the viability of the organisms and for maintaining the final conditions towards which cultures of maximum population tend.

3. *The Variation among Individuals Inhabiting Cultures of Infusoria.*

It has been shown by Jennings (9) that the individuals inhabiting a culture of *Paramoecia* are not morphologically alike, and that the morphological differences can be perpetuated through a large number of generations.

It is evident from the results obtained by Cutler and Crump with *Colpidia*, and from the results enumerated above, that the individuals inhabiting a culture of infusoria also differ among themselves physiologically, and that these differences are evidenced by differing sensibility to adverse conditions, i.e., in the above

experiments, to an unfavourable reaction of the medium. Not all of the individuals which inhabit the culture die when the reaction becomes unfavourable, but only a proportion of them. While in rapidly multiplying cultures, as in the experiments described above, the physiological differences underlying differences of sensibility to toxic agents might conceivably originate in differences of age, it has also been found, both by Cutler and Crump and by the author, that similar partial mortality occurs in adult cultures in which multiplication is no longer taking place.

It has been shown by Peters that similar differences occur in the sensibility of the individuals comprising an infusorial population towards the toxic effects of antiseptics, and he suggests that if similar differences subsist between bacteria, the distribution of their susceptibilities in a frequency-curve would lead to time-relations of disinfection similar to those which characterize a monomolecular chemical reaction (11). He overlooks the fact, however, that the monomolecular law itself originates in a similar chance distribution of instability in the reacting molecules (or else of their situation in space), from which, as the author has shown, the monomolecular law of disinfection may also be deduced (13). The experiments of Peters, therefore, do not reveal the origin of the monomolecular law of disinfection, but merely the impossibility of deciding, from the time-relationships alone, whether the distribution of deaths in time is primarily attributable to the fortuitous distribution of molecules in space or to the fortuitous distribution of susceptibility in the organisms. It is evident, however, that differences of susceptibility do obtain between the individuals which comprise a population of infusoria.

The existence of such differences, capable, moreover, of affecting the multiplicative rate in subcultures, is revealed by the following facts:

If a large number of subcultures are prepared from a single parent-culture, whether the isolated individuals are washed or not, it is very frequently found, on warm days, that the last subcultures display a much higher reproductive rate than the earlier ones. This is due, however, to the greater viability of the later cultures, and not to differences in the reproductive rates of survivors, as the following experiments reveal:

Experiment 1. Nine subcultures taken from a parent-culture 5 days old; the organisms were not washed:

Serial Number of Subculture.	Initial Number of Organisms.	Number contained in Cultures after 18 hours.	22 hours.	26 hours.	42 hours.
First	8	2	2	2	6
Ninth	6	6	6	6	13

Experiment 2. Seven subcultures taken from a parent-culture 4 days old; the organisms were not washed:

Serial Number of Subculture.	Initial Number of Organisms.	Number contained in Culture after 19 hours.	23 hours.	26 hours.
First	14	5	5	7
Seventh	7	7	10	10

Experiment 3. Eight subcultures taken from a parent-culture 4 days old; the organisms were not washed:

Serial Number of Subculture.	Initial Number of Organisms.	Number contained in Cultures after 18 hours.	22 hours.	43 hours.
First	1	0	0	0
Second	1	0	0	0
Eighth	1	1	1	15

Experiment 4. Nine subcultures taken from a parent-culture 3 days old; the organisms were washed once:

Serial Number of Subculture.	Initial Number of Organisms.	Number contained in Cultures after 19 hours.	26 hours.
First	4	3	5
Ninth	4	4	10

Experiment 5. Nine subcultures taken from a parent-culture 3 days old; the organisms were not washed:

Serial Number of Subculture.	Initial Number of Organisms.	Number contained in Cultures after 18 hours.
First	26	0
Seventh	29	138
Eighth	31	327
Ninth	25	185

Numerous results of the same character were obtained, but those reported show the distinctive features of the phenomenon. The lag-period is not affected. The reproductive rates of those individuals in the earlier cultures *which chance to survive* are not diminished, but the mortality in the early cultures is much higher than it is in the later cultures. It will be recollected that this is only true, as a rule, in hot weather (temperature 25° to 30°C.), provided that the hay infusion is initially brought to a P_H lying between 7·3 and 8·3 by the addition of bicarbonate or sodium hydroxide in suitable proportions.

In experiments designed to compare the effects of successive modifications of or additions to the culture medium upon the reproductive rate of the organisms, the phenomenon, when it occurred, was very troublesome. Whenever experience showed that it was likely to occur the precaution was taken of culturing into normal hay infusion at the beginning and at the end of each series of subcultures. If the apparent reproductive rates in the first and last subcultures were then found to differ materially, the entire experiment was rejected.

The above-cited results were obtained in experiments which were rejected for this reason.

Another way of overcoming the effect which was frequently adopted was that of diluting a small proportion (0·003 to 0·005 cc.) of the parent-culture with from 20 to 25 times its volume of fresh hay infusion, and isolating all the subcultures from this sample. The subcultures were then almost uniform in their behaviour.

Reviewing the technique of the operations leading to the isolation of individuals to form subcultures, three possible origins of the phenomenon suggested themselves, namely:

(*a*) Exposure to the strong artificial light employed in counting the isolated infusoria, and which was allowed to fall upon the parent-culture during the time occupied in isolating subcultures. At other times the cultures were only exposed to dim diffused daylight.

(*b*) Evaporation from the surface of the culture after removal of the cover of the Petri dish in which it was kept, leading to an increase of osmotic pressure of the parent-culture.

(*c*) Reduction of the volume of the parent-culture by repeated removal of samples. The samples removed were frequently more numerous than the subcultures, because certain samples, containing too many or too few organisms, were always rejected.

Direct experiment showed that (*a*) was not the origin of the effect. A sample was withdrawn from a culture; the individuals in it were counted, and a subculture formed from them. The parent-culture was then exposed to the strong light for an hour, and another sample withdrawn, counted, and subcultured. In every case the viability of the two cultures was the same. It might be suggested that this experiment also ruled out possibility (*b*), since the cover of the Petri dish was removed to allow the light to play directly upon the drop containing the culture. But evaporation would obviously be more rapid the smaller the drop, so that as the size of the drop was reduced by successive withdrawals of samples, the increase in osmotic pressure might possibly be considerably greater than that arising from exposure of the undiminished drop to free evaporation for an hour. It still remained possible, therefore, that the origin of the phenomenon resided in a combination of the factors (*b*) and (*c*).

A number of experiments were carried out, therefore, in which concentrated Ringer solution was added, in small proportion, to the hay infusion, in order to increase its osmotic pressure without diluting it to any important extent. In some experiments the organisms were directly subcultured into these media. In others they were immersed in the hypertonic media for varying periods, and then transferred to normal hay infusion, forming the subcultures.

It was found that *Enchelys* is remarkably resistant to hypertonicity arising from inorganic salts. On first introducing the organisms into these hypertonic

media the withdrawal of water was very manifest, and the organisms were compressed laterally into disc-shaped bodies. Within five or ten minutes at the utmost, however, in concentrations of added salts varying from $\frac{1}{16}$ the concentration of mammalian Ringer to its full concentration, the organisms regained their normal contour, although, in the highest concentrations (full strength added Ringer) the organisms ultimately died. Cultures in hay infusion containing half-strength added Ringer ($= \frac{M}{12}$ sodium-chloride) sometimes survived without diminution of number for 48 hours, but no divisions occurred. In lower concentrations of added Ringer the organisms not only survived, but underwent reproduction at a rate only slightly less than normal. The following is an illustrative experiment:

Parent-culture 3 days old; temperature 25°C

Culture No.	Concentration of added Ringer.	Number of Infusoria produced per initial individual after 18 hours.
280A	None	18
281A	None	22
282A	M/96	15
283A	M/48	16
284A	M/24	15
285A	M/12	0
286A	M/6	0
287A	None	22
288A	None	18

No improvement of reproductive rate or viability was observed in any of the hyptertonic media. The effect of evaporation of the parent-culture during the processes of subculturing, however, would be to expose the organisms for a while to a hypertonic medium, followed by return to a normal medium, now hyptonic in comparison with the medium to which they have become adapted. Experimental imitation of these conditions, however, yielded uniformly negative results. In no case was the viability of subcultures improved; on the contrary, if the hyptertonic media contained from $\frac{M}{12}$ to $\frac{M}{6}$ added Ringer, exposure for 5 or 10 minutes to these concentrations followed by return to normal hay infusion, usually resulted in death of all the individuals in the subculture. Exposure of similar duration to less hypertonic solutions had no effect whatever upon the reproductive rates or viability of the organisms.

It appears, therefore, that we must attribute the superior viability of late samples withdrawn from the parent-culture primarily to factor (*c*) enumerated above.

Low and slow-swimming organisms, usually of a more spherical outline than those which swim actively and high in the drop, are difficult to withdraw from

a drop of 0·1 cc. volume in a sample of 0·003 to 0·005 cc. An extreme example of this is afforded by the cysts of the large infusorian employed in experiments reported in previous articles (17, 18).[4] These lie at the bottom of the drop, and a sample withdrawn from it contains, as a rule, no cysts at all, or far less than the due proportion of cysts. The cysts themselves are sticky, and adhere somewhat to the bottom of the slide, but the statement remains true, even when the cysts are detached by rubbing the bottom of the slide with a pointed glass rod. Moreover, for a considerable period prior to actual encystment these organisms lie quiet at the bottom of the drop, and these are equally difficult to withdraw in the first samples. Only when the drop becomes so diminished in volume that these low-swimming forms are densely crowded do they appear in their due proportion in the samples withdrawn.

Enchelys similarly, but to a much less degree, varies in its form and habit of swimming, and low-swimming forms are usually of the well-nourished, spherical type described in a previous communication (14). For this reason they are ill-represented in samples withdrawn from a large drop, and it is probable that the greater viability of the individuals withdrawn in the later samples is due to the contraction of the dimensions of the drop and the greater proportion of low-swimming individuals which are withdrawn in the sample. Evidently, in an adult culture, such individuals do not differ from the high-swimming type in reproductive rate or lag, but they differ from them in the superiority of their resistance to the adverse conditions which may arise during the first 24 hours in newly inoculated cultures.

SUMMARY.

1. Allelocatalysis (mutual acceleration of reproduction by contiguous cells) is displayed only between pairs of *living* infusoria. It follows from this that if the conditions of inoculation or the character of the medium are such as to lead to an extensive mortality among the individuals which form the cultures, allelocatalytic effect must be obscured, and that if the mortality attains to 50% it must fail altogether to be displayed.

2. Subcultures of infusoria *(Enchelys)* which are isolated into hay infusion almost always die at temperatures in the neighbourhood of 30°C. Two means have been found of ensuring the viability of these subcultures, provided that the temperature at which they are maintained does not too greatly exceed the limit of 30°C. One consists in pre-cooling the parent-culture to 4°C. for 24 hours preceding the abstraction of individuals to form the subculture. The other consists in allowing the culture fluid to stand for 24 hours at room temperature before subculturing into it.

[4]This organism was submitted for identification to Dr. J. S. Dunkerly, who very kindly informs me that it is *Colpoda cucullus* (Miller).

3. Hay infusion and the synthetic culture medium employed by Cutler and Crump have been titrated electrometrically. It was found that whereas they are equally buffered between the P_H values of 6·4 and 7·8, the synthetic medium is much less effectively buffered against excess of acid or alkali than hay infusion for P_H values below or above these limits.

4. The changes in P_H, bacterial count, and infusorial population of subcultures at 23°C. have been investigated. It is found that the P_H of subcultures, whether these are initially alkaline or initially neutral, at first falls and later rises, ultimately reaching a stable value in the neighbourhood of 8·0 to 8·3. During the period of relative acidity the infusorial population remains stationary or diminishes. Multiplication is resumed as the reaction becomes more alkaline. The multiplication of bacteria, on the contrary, is unaffected by these changes in the reaction of the medium.

5. The members of a thickly-inhabited infusorial population differ among themselves in their viability when transferred to freshly prepared culture medium. The low-swimming individuals of more spherical outline are most resistant to the adverse conditions which may arise at some time during the first 24 hours in subcultures. The lag-period and reproductive rate do not vary to nearly so great an extent among different individuals as their viability after isolation.

6. *Enchelys* is remarkably resistant to hypertonicity arising from inorganic salts. Cultures in hay infusion containing added Ringer solution equivalent in concentration to $\frac{M}{12}$ sodium-chloride sometimes survive without diminution in number for over 48 hours, but without undergoing division. Viability is not improved by either brief or prolonged exposure to hypertonic media. Brief exposure to hypertonic media does not affect the subsequent duration of lag or the reproductive rate, provided the hypertonicity ($\frac{M}{12}$ added Ringer or over) is not sufficient to bring about the death of the organisms which are exposed to it.

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A FURTHER NOTE ON THE STRUCTURE OF THE NEURONE JUNCTIONS OF THE SPINAL CORD

by

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(Submitted for publication 7th January, 1927.)

In previous papers in this journal I have pointed out that there is no unquestionable evidence available for the conception of neurones terminating upon the body of nerve cells in the spinal cord by ramifying synapses which embrace the body of the nerve cell. On the contrary, it was concluded that the neurone junctions take place via the cell dendrites. The collaterals, entering the grey matter from higher or lower levels of the cord undergo extensive branching in the grey matter, and these branches, travelling in various directions within the grey matter, enter, either singly or in conjunction with the branches of other collaterals, one or other of the numerous dendrites of the nerve cells.

It has hitherto been impossible to bear this statement out by means of direct observation, since it is practically certain that the nerve cells which form part of a reflex arc will not be included in their entirety within a section of the cord of the thinness necessary for microscopic observation. The evidence on which the statement is based is as follows:

(*a*) The neurofibrils of the nerve cells leave the body of the nerve cells, and, having entered the dendrites, emerge from these, and travel for long distances as naked neurofibrils in the grey matter of the cord. No real termination of these fibrils can be revealed after they emerge from the nerve cells, since their ending is only an apparent one, due to the fact that they are cut short as they leave the plane of the section.

(*b*) In preparations made by the reduced silver method, in which apparently the nerve fibres have stained almost in their totality, no indication of branching synapses can be detected (10). This might be taken as valueless negative evidence, since it might be urged that the branching "synapse" had a different staining reaction from the rest of the nerve fibre, so that the terminal part of the fibre would not be rendered visible. This objection is disproved by the fact that it would reveal an obvious discontinuity of the fibres at the points where the staining had ceased; but such discontinuity, as already stated, does not exist.

(*c*) When the collaterals are traced from the white into the grey matter they are found to undergo branching; but these branches cannot be shown to have any relation to the nerve cells of the grey matter, for sooner or later they always emerge from the plane of the section. But these nerve fibres have precisely the same appearance as the naked neurofibrils that emerge from the cell dendrites, and since these latter also cannot be shown to have any ending, and since no branching synapses have been demonstrated, it is concluded that the collaterals are one and the same thing as the naked neurofibrils that leave the nerve cells through their dendrites.

Since making these observations it occurred to me that there was a simple means available for recognizing collaterals within the grey matter, quite apart from tracing continuity between them and a definite axon. This depends on the fact that a collateral usually undergoes a certain amount of branching within the grey matter; sometimes, indeed, the branching is considerable. A branching collateral may usually be recognized by the fact that at the point of branching there is a small triangular piece of protoplasm, and this piece of protoplasm is so located, also, that it enables us to recognize the direction in which the fibre is travelling. Sometimes branching of neurones occurs without such a distinguishing feature, but these fibres have never been used as a means of recognizing collaterals in this investigation, since a certain amount of uncertainty always attaches to their identification in cases where we are dealing with two fibres that run one above the other for a considerable distance, and then suddenly separate. The object of the investigation was to find collaterals, recognizable by this feature, which, even if they could not be traced outwards in the white matter, yet could be traced into the cell dendrites. The making of the observations is attended by a great deal of difficulty. Thick and fairly completely stained sections are necessary; when the point of branching of a collateral is detected the two branches are followed in the direction in which the fibre is running. Very frequently it is impossible to trace these branches far with certainty, since they become confused with other fibres, and it is practically impossible to follow them individually with certainty; but much more frequently, sometimes after they have travelled a short distance, at other times after one has successfully followed them for very long distances, they will gradually or suddenly bend, and move out of the plane of the section. In fact, hundreds of attempts to follow these collaterals are but awarded with one or other of these exasperating results. But occasionally I have actually succeeded in tracing a branch from these collaterals into the cell dendrite.

Three examples of this are shown in figures 1, 2, and 3, taken from the spinal cord of a young rabbit, and prepared by Cajal's reduced silver method. When examining the branching collaterals, two types of branching may be distinguished: there are those cases where the fibre divides into two branches of approximately equal thickness, or there are cases where one of the branches is of extreme delicacy, with a diameter even smaller than that of most neurofibrils;

REFERENCE TO PLATE.

Figures 1 and 2. Two anterior horn cells from rabbit (Cajal reduced silver method). A collateral undergoes branching at X, and one of the branches penetrates the dendrite of the anterior horn cell and becomes identical with one of its neurofibrils. a - - - axon.

Figure 3. Posterior horn cell, showing the same.

Figure 4. A nerve cell from spinal cord of unborn rabbit, unfixed, and bathed in amniotic fluid. An attempt has been made to represent the individual fibrils as accurately as possible.

and it is a particularly difficult matter to follow this latter type of fibril for any considerable distance. In figure 1 is shown a case where a very delicate collateral divides into two extremely delicate branches, and one of these enters a dendrite of an adjacent nerve cell; the other branch emerges from the plane of the section. In figure 2 is shown a collateral giving off a considerably thicker branch to an anterior horn cell. Figure 3 shows a similar case from a cell of the posterior horn, closely adjacent to the substance of Rolando. The fact that only a small number of such cases has been found, while on the other hand every nerve cell that is at all well stained shows numerous neurofibrils emerging naked from the dendrites, might be taken to mean that little importance can be attached to this mode of neurone junction. The criticism would be unjust, for in the majority of collaterals that one observes, the manner of branching is such that it would be impossible to reveal their actual junction with the nerve cells.

From this direct observation we are therefore able to conclude that the predominant type of neurone junction in the spinal cord is a direct junction via the cell dendrites. Whether a second type of neurone junction in the form of branching synapses applied to the cell body occurs cannot be stated at present. The reduced silver method fails to reveal them. The terminal "buttons" which Cajal has described by this method cannot be accepted without further evidence; they have never been shown to be the terminations of undoubted nerve fibres Similar effects may often be obtained, especially in older animals, at the surface of nerve cells which have undergone marked shrinkage, and the fibrils appear to represent coagulated intercellular matrix drawn into fibrils during the shrinking of the cell. On the other hand the possibility has not been excluded that they may be terminations of neuroglia fibres. Golgi observed many years ago that neuroglia fibres may end in this manner upon blood vessels, and Cajal gives many illustrations of the same. In addition to the observations recorded in a previous paper (9), I have made numerous examinations of spinal cords stained intra-vitam with methylene blue, but have never observed a true synapse. It was by this method that Cajal believed he had rendered such a synapse visible. The observation is, however, open to question. Cajal remarks (5, p. 312), that while the Weigert-Pal and Ehrlich methods are capable of revealing the collaterals in the region where these branch off from the longitudinal fibres of the white matter, these methods are quite unsuitable for tracing collaterals far into the grey matter of the cord. Nevertheless, it is with this admittedly defective technique that the most convincing example of a synapse has been described. Until the observation can be confirmed by others it cannot be accepted. In numerous methylene blue preparations, prepared by Cajal's diffusion method, or by Dogiel's immersion process, I have failed to obtain any indication of them. I find, on the contrary, that the surface of the nerve cells and the intercellular matrix of the cord (especially the latter) may undergo an extraordinary amount of "frothing" and disorganization during the preparation of the material; this is in the main

probably due to the fact that no true fixation of the tissue occurs. While methylene blue is an excellent stain for such peripheral plexuses as the Auerbach plexus, it cannot at present be so satisfactorily applied to the central nervous system. Much seems to depend on the type of methylene blue employed. I have used the "med. pur." and the "Grübler nach Ehrlich" samples, as well as several other less known ones, but while these give excellent results with the intestinal plexus, they have been practically useless for the spinal cord. Satisfactory staining of the nerve cells is usually easily obtained, but the finer nerve fibres do not react at all well; but even in cases where the fine nerve fibres of the grey matter have been partially stained, no trace of synapses can be seen. On the other hand, the artefacts obtained by this method are so extensive that it is often possible to see almost anything into the preparations.

By means of the Golgi method Cajal has observed and figured numerous instances of collaterals breaking up into small bunches of delicate fibrils. Most of the illustrations to chapter XI of his book would serve as examples. None of these cases, however, affect the point at issue; that the collaterals undergo extensive branching is not, of course, denied, but that these bunches of fine fibres are terminal synapses does not by any means follow. None of the branches are actually shown terminating upon the nerve cells, though they are certainly mostly in the neighbourhood of the nerve cells. Even this does not always hold true; for instance, in figure 111 of his book many of the collaterals appear to terminate in the region of the substance of Rolando, which is devoid of nerve cells, and even in the posterior horn one does not find by any means as numerous nerve cells as the number of nerve terminations would lead one to expect.

The conclusion that we seem justified in drawing from these observations is that the majority of neurone junctions in the cord occur by collaterals directly penetrating the dendrites of the nerve cells, and becoming continuous with their neurofibrils, and that the evidence for the existence of synapses is inadequate. Even if cases of fibrils terminating on the body of nerve cells be discovered in the spinal cord, the contact theory will still be doubted. Thus F. M. Ballantyne (2), using the lung-fish (Lepidosiren) as material, has recently discovered and figured nerve fibres which undergo a small amount of branching near the body of the nerve cells, and having penetrated the body of the nerve cell, become identical with its neurofibrils.

These observations, moreover, cast doubt upon the theory of contact rather than of continuity, as applied to the remainder of the central nervous system. Those cases in which definite discontinuity has been observed are largely deprived of significance, in that discontinuity may be an artificial result of shrinkage of the nerve tissue occurring during its preparation. Attention may be called to the fact that in one case of nerve termination, namely, the ending of a motor nerve upon a "striated" muscle fibre, we have continuity and not contact merely. Such a nerve termination does not seem to differ essentially from the termination of one neurone upon another: embryologically they are similar; if the nerve

fibre in either case be sectioned degeneration does not primarily proceed beyond the ending of the nerve fibre, and degeneration of the next nerve cell, or of the muscle fibre, is a secondary and much delayed event, due to disuse apparently, and not to its isolation. Other points of similarity in the two are a marked slowing of the rate of conduction at the junction and a readiness to undergo fatigue. In this case of motor nerve endings upon "striated" muscle fibre, a case for which a fairly satisfactory technique has been worked out, contact rather than continuity has frequently been described. Nevertheless, the recent work of Boeke (3) shows beyond all question that true continuity does occur.

Histological research leads us to the conclusion that the neurofibril, rather than the neurone, is the elementary conducting unit of the nervous system. Against this conception has frequently been raised the objection that the neurofibril cannot be observed in living nerve cells, and is merely a post-mortem coagulation product. This denial of their existence, a view maintained by F. W. Mott and by Marinesco, loses its significance when we recollect that uncoloured structures will only be visible within nerve cells if their refractive index differs markedly from that of the cytoplasmic fluid which bathes them; a microscope slide, standing in a beaker of water, is invisible except at its rough edges (where the light is irregularly reflected), but it would not be justifiable to deny the presence of the glass slide merely because it cannot be seen. Boeke, on the contrary, remarks that in the nerve cells of the leech, treatment of the nerve cells with very dilute methylene blue, which does not coagulate the protoplasm, results in the staining of these neurofibrils (4). Even if methylene blue were toxic for the nerve cell, it would scarcely be expected to bring about the same coagulation picture as such a substance as alcohol, which is used in the Cajal reduced silver technique. Nevertheless, the two methods give similar results.

In contradiction to the view that the neurofibrils are artefacts, I may remark that I have frequently observed a faint fibrillary structure in unfixed and apparently living nerve cells from young rabbits, bathed in their own body fluids. That the fibrils cannot be seen with a clearness in any way approaching that of the fixed and stained preparation is true, but the fact that a fibrillation may at times be detected is itself strong evidence that the neurofibrillae are not mere fixation products. Such a case of fibrils within an unfixed nerve cell is shown in figure 4. It must be admitted, at the same time, that usually no trace of fibrils is to be seen within the living nerve cells.

In addition to the observation of Boeke on the nerve cells of the leech, it may be recalled that Max Schultze observed a fibrillary structure in living nerve cells bathed in serum, and both Apathy (1), and especially Göthlin (6), examining the living nerve cells of various vertebrates and invertebrates by means of polarized light, have established the existence of neurofibrils in unfixed nerve cells. Similarly, Howard (7) records the presence of neurofibrils in the fresh unfixed visual cells of vertebrates. These various observations seem to show that

a system of fibrils, even if indistinct, can be revealed within the body of unfixed living nerve cells under favourable conditions.

It may at the same time be pointed out that if a portion of fresh spinal cord of a young rabbit be examined in serum, while it is true that the neurofibrils are rarely (and then only indistinctly) visible, so also it is usually impossible to detect any trace of the enormous number of extremely delicate collaterals which the reduced silver and Golgi methods demonstrate in the grey matter. It is of further interest to note that in not a single nerve cell examined fresh in serum is any trace visible of the numerous synapses that are supposed to be present on its surface. The microphotographs of Marinesco (8) also reveal no trace of them. It does not therefore seem justifiable to deny the existence of neurofibrils in living nerve cells, when we certainly have good evidence for their existence in such cells, and at the same time uphold the thory of numerous synapses embracing the body of the nerve cells, when the evidence for their presence is so very scanty.

SUMMARY.

1. Collaterals are recognizable within the grey matter of the spinal cord by the fact that they undergo branching, the point of branching being marked by a small triangular piece of protoplasm.

2. Most of the collaterals thus identified, when followed along their length, emerge from the plane of the section, or become confused with other fibres.

3. But occasionally it is possible to trace these fibrils right into the dendrites. This is taken as direct evidence in support of the view previously put forward on indirect evidence, that there is a direct neurone continuity in the spinal cord, the collaterals entering the nerve cells via the cell dendrites.

4. No evidence for the existence of synapses branching on the outside of the nerve cells has yet been found.

5. In support of the view that the neurofibrils are genuine cell structures, it is pointed out that under certain circumstances they may be seen in the unfixed nerve cell.

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THE SUPPOSED DISTINCTION OF AUSTRALIAN ABORIGINALS INTO "STRAIGHT" AND "WAVY"-HAIRED INDIVIDUALS

by

O. W. TIEGS.

With a Foreword by Sir W. Baldwin Spencer.

(Submitted for publication 10th February, 1927.)

The Arunta tribe is divided into two exogamous intermarrying moieties, each of which again, in the northern part of the tribe, is divided into two classes. Those of the one moiety are called Panunga and Bultara; those of the other Purula and Kumara. In some tribes there are distinct names for the moieties, such as Dilbi and Kupathin in the Kabi tribe, Queensland, Mukwara and Kilpara in the Barkinji tribe, New South Wales. Of these the first two are said to signify respectively light and dark blood, the second two either straight and curly hair, or by some authors, eagle hawk and crow.[1] The Euahlayi tribe in New South Wales is divided into Gwaigulleah (light-blooded) and Gwaimudthen (dark-blooded) men and women.[2] In some tribes of South-west Australia the class system is said to be based on colours—white cockatoo and crow, light and dark purple.

The Arunta have no names for their moieties, but the Panunga-Bultara people are called *Gola aradjina* (straight-haired) and *Mberka oknirra* (big-bodied); the Purula-Kumara, on the other hand, are known as *Gola luda-luda* (wavy or curly-haired) and *Mberka tungwa* (small-bodied). These are not used as moiety names, but at the same time they indicate clearly the native belief that the tribe is composed of two groups, distinguished from one another by marked physical characters. It must be pointed out that the term *luda-luda* does not refer to a curly form, such as that of the hair of negroid people or the lost Tasmanians, nor does *aradjina* refer to a straight form, such as that of Malays. In fact, to the ordinary observer there is not the slightest difference to be detected between the two forms, and a native only distinguishes between them, or attempts to do so, after minute examination of individual hairs. The examination to which the native subjects the hair is not directed towards detecting a difference in the

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[1]Rev. John Mathew, Journal R. Anthrop. Inst., vol. xl, p. 165.

[2]The Euahlayi tribe; Mrs. Langloh Parker; p. 11.

texture of the hairs, as is done, for example, by wool classers, but consists, on the contrary, in holding a hair, drawn straight out, up to the light and observing the presence or absence of waviness in its actual appearance. In a test to which I submitted the native who gave me the information, it was quite evident that he thought he could distinguish in one set of hairs a minute wavy structure, absent in the other, but, neither with the naked eye nor with the aid of a hand lens, could I distinguish any difference. Out of twenty-two that came from the *Panunga-Bultara* moiety, and, on the native theory, ought all to have been *Aradjina*, he identified seventeen as such and five as *Luda-luda;* out of seventeen that came from the *Purula-Kumara* moiety, and ought all to have been *Luda-luda*, he identified nine as such and eight as *Aradjina;* that is, out of a total number of thirty-nine, he was right in twenty-six and wrong in thirteen cases.

W.B.S.

When the hairs of the *Luda-luda* and *Aradjina* divisions are held up to the light and examined it is not possible to detect any difference between the two types, since, on account of the thinness of the individual hair, we cannot be certain of any change in the diameter of the hairs along their length. But when these hairs are examined under a low-powered microscope the impression is frequently gained of long waves in the hair, in the form of alternating regions of thickness and thinness. Measurement of the diameter of the hairs at various points along their length confirms this observation, and it is found that while some hairs are devoid of such waves, others show them markedly.

Hairs with or without a wave may occur upon the same individual, and of twenty hairs whose thickness was carefully measured at points 2 mm. apart along their length, five showed but little indication of a wave, and might be classed as "straight," while fourteen showed a pronounced wave, and one was intermediate between the two. Of the fourteen which showed a wave, some showed it much more markedly than did others. In fact, while some hairs are definitely "straight," and others are definitely "wavy," all intermediate gradations occur; and hairs are often found one part of which is "wavy" and another practically "straight." Of the five hairs which were "straight," three belonged to the *Aradjina* division, two to the *Luda-luda*. Of the fourteen which showed a wave, ten were *Aradjina* and four were *Luda-luda*. The intermediate one was an *Aradjina*.

The following measurements of four hairs, two from individuals belonging to each division of the tribe, as recognized by the natives, will serve to show that in both of them each type of hair occurs:

Luda-luda:

(*a*) 28, 23, 22, 27, 25½, 25, 21, 18, 22, 21¼, 17, 20, 18, 18, 15, 15, 16 (i.e., wavy).

(*b*) 20, 20, 20, 20, 20, 20, 21, 21, 21, 21, 21, 21, 21, 20, 20, 20, 20, 20, 20, 20, 20, 20, 20, 20 (i.e., straight).

Aradjina:

(*a*) 21, 20½, 20½, 20, 20, 20, 20½, 20½, 21, 20½, 20½, 20½, 20, 20, 20½, 20, 20½ (i.e., straight).

(b) 27, 21, 21, 20, 21, 20, 20½, 17, 13, 18, 20, 21, 20½ (i.e., wavy).

The numbers are micrometer measurements, and each unit corresponds to ·006μ. The waves are of irregular length, usually about a centimeter long, sometimes shorter, often much longer.

It is possible that by *Luda-luda* the native understands, not an alternating series of thick and thin regions, but a faint wave in the contour of the line of the hair in one plane, i.e., a faint indication of a zig-zag line. No indication of such a condition can be detected with the microscope.

While it may be definitely stated that wavy hairs of the type which the aboriginal thinks he observes do not occur, yet it is possible that two different types of hair may occur, in one of which there are alternating regions of darker and lighter pigmentation, giving the appearance of regions of thickness and thinness; or alternating segments of a hair may possess different optical properties, which might be similarly interpreted by the aboriginals, even if they were not visible, on account of the fineness of the hair. Such hairs might be interpreted as wavy hairs, and the aboriginal belief might be founded on fact. Such a "wave" would probably not be detectable by our ordinary methods of examining hairs, mounted on slides, in transmitted light, but should become clearly visible if a hair is held up to the light, and examined with a low-power binocular lens. I find that if this test be carried out, using a lens magnifying about three diameters, nothing that might be interpreted as waves of the kind referred to by the natives can be detected. It would appear, therefore, that the classification of individuals into "straight" and "wavy" groups is unjustified.

It is difficult to define precisely what the term *Luda-luda* means, as applied by the native to the structure of the hair, because whilst he uses this term to describe a wavy line, he also uses it for a zig-zag line. Possibly what we call wavy is not, in this case, identical with what the native calls *Luda-luda*, and certainly the latter and *Aradjina* imply to him a difference in structure that does not actually exist as distinctive of the two main divisions of the tribe. Such differences as do exist between the two types of hair are due to variations in structure so minute that they could not possibly be detected by the naked eye. Moreover, all such variations occur not only in the hair of the same individual, but in different parts of the same hair.

Concerning the belief of the aboriginals that they are composed of a wavy- and a straight-haired race, it may be pointed out that there is evidence to show that a mixture of "blood" has occurred. Thus it has recently been found by

Cleland (1) that two distinct blood-groups occur in South Australian aboriginals. This suggests that the widespread idea of the aboriginal that he is composed of two distinct races, still recognizable by their straight and wavy hair, is a tradition which has descended from a past age, when a wavy- and a straight-haired race existed, and that the two races have now largely fused. It does not seem as if much weight can be attached to the observation that some hairs are waved in the manner above described, and others, from the same individual, not (with the possible inference that this represents a fusion of straightness and waviness), since, in the first place, this kind of wave is one that the naked eye could never detect, and, secondly, similar waves can be found in the hairs of Europeans. For example, the hair of a European, measured at intervals of 2 mm. along its length, gave the following thicknesses: 18, 14, 15, 17, 17, 15, 16, 16, 16, 14, 13, 13, 12, 15, 15, 14, 15, 15, 14, 14, 15, 15, 14, 12, 13, 12, 12, 13.

The aboriginal's belief that he can recognize two different types of individuals, having respectively straight and wavy hair, cannot therefore be confirmed. But it is of interest to observe that when a hair is drawn straight out and suitably held, there occurs a play of light upon its surface. This may produce an ill-defined, dazzling effect, into which the imagination may read whatever it desires, and it probably depends upon whether the native sees waviness or straightness into the ill-defined line that he classes his individuals as *Luda-luda* or *Aradjina*.

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THE ORIGIN OF ACETALDEHYDE IN BACTERIAL AND ANIMAL METABOLISM

by

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(Submitted for publication 14th January, 1927.)

1. *The Pyruvic Acid-Acetaldehyde Theory of Carbohydrate Breakdown.*

The pyruvic acid–acetaldehyde theory of carbohydrate breakdown, established for normal yeast fermentations by the work of Neuberg and his colleagues, has been applied also to carbohydrate breakdown by other living cells of all kinds. [See, for example Neuberg in Oppenheimer's *Handbuch der Biochemie*, 2nd ed., vol. II, and Embden (12).] In the case of bacterial and animal cells, the evidence appears to need further examination. This paper attempts such examination, and adduces some bacteriological evidence which has not previously been cited in this connection, but which seems apposite.

Acetyl methyl carbinol as an indicator of the production of acetaldehyde by bacteria.

Neuberg and his school have usually demonstrated the occurrence of acetaldehyde in fermentations and excised tissues by means of fixatives (sulphites and dimedon). A natural indicator of the presence of acetaldehyde during growth of a micro-organism is available in some cases in the substance acetyl methyl carbinol or acetoin $CH_3.CHOH.CO.CH_3$, formed by the simple condensation of two molecules of acetaldehyde. The power to make this substance, correlated by Neuberg with the presence of an enzyme called by him carboligase, is possessed by yeast (see Neuberg and Hirsch, 63), and by a number of micro-organisms, including *B. subtilis*, *B. lactis aerogenes*, *V. cholerae*, *B. diphtheriae*, and others. That acetoin originates from acetaldehyde appears now to be generally accepted. When formed it is remarkably inert. It may undergo reduction to 2.3.butylene glycol, $CH_3.CHOH.CHOH.CH_3$, or possibly oxidation to di-acetyl, $CH_3.CO.CO.CH_3$, but there is no evidence that it can be further oxidized by micro-organisms to furnish energy. Meyerhof (50) has shown that it does not increase the oxygen uptake of yeast, and the carbon-dioxide output of yeast is

also unaffected by it (Neuberg and Kobel, 64). Di-acetyl or acetoin can be added to a yeast fermentation and recovered again mainly as butylene glycol (Neuberg and Nord 67, Neuberg and Kobel 64). The present writer has followed the course of production of acetoin and butylene glycol from sugar by a soil bacillus, and has found that the sum of the two reaches a maximum at the time when the last of the sugar disappears, and does not subsequently diminish.

To use acetoin as an indication that acetaldehyde has been formed in the course of a fermentation is perhaps better than adding sulphite as a fixative. The normal balance of reactions is not altered; that this may be a real danger in the presence of sulphite is suggested by the work of Bleyer and Schmidt (6) on the behaviour of sugars in sulphite-containing solutions. No large accumulation of acetaldehyde is necessary for acetoin to be formed; traces can be demonstrated in well-aerated normal yeast fermentations. When formed it is stable, and easily detected in minimal quantities by the method devised by Lemoigne (36), and improved by Kluyver, Donker, and Visser t'Hooft (34). The principle of the method is oxidation of acetoin to diacetyl, and precipitation of di-acetyl as nickel-dimethyl-glyoxime.

Examining yeast as a test case, it is found that observations on acetoin and on sulphite fixations give concordant results. Traces of acetoin may be present in normal fermentations; the quantity can be increased by aeration or by adding hydrogen acceptors, such as methylene blue, sulphur, or more acetaldehyde; less acetaldehyde is then reduced to ethyl alcohol, and more remains to be condensed into acetoin. In alkaline fermentations, parallel with a small accumulation of acetaldehyde, runs a formation of acetoin. The action of yeast on pyruvate leads to the production of acetoin in large quantities. (See papers by Neuberg and his collaborators, in the Biochemische Zeitschrift, from 1921 onwards.)

When the acetoin-producing bacteria are investigated with regard to acetaldehyde production, it is found that acetoin (and one may conclude acetaldehyde) is formed freely from sugars and glycerol, but only sparingly from lactate and pyruvate. The experiments of Harden and Norris (27) show that *B. lactis aerogenes* forms acetoin or butylene glycol from a variety of sugars, from glycerol, and from acetaldehyde. Ethelyene glycol also gives rise to butylene glycol, the preliminary reaction being, one may suppose, the removal of the elements of water to leave acetaldehyde:

$$CH_2OH.CH_2OH \longrightarrow CH_3.CHO + HOH.$$

No acetoin was formed from citrate or malate. A peptone medium was used throughout. Lemoigne (35) found acetoin after growth of *B. subtilis* on peptone-sucrose or peptone-glycerol media, but it was not produced in a peptone-lactate medium. Later (37) he succeeded in obtaining minute quantities of acetoin after long continued growth of an organism of the *subtilis* group on a calcium lactate-peptone medium. Observations by the present writer on the acetoin-forming soil bacillus previously mentioned show that it does not make acetoin from lactate

or pyruvate, in spite of excellent growth, while from a 2% glucose medium up to 10% of the sugar may be recovered as acetoin and butylene glycol. Acetoin is also formed from glycerol, but not from succinate. In view of the increased yield of acetoin obtained by aeration of yeast fermentations, the soil bacillus was cultivated on lactate and on pyruvate in a stream of oxygen, but still not a trace of acetoin could be detected by the very sensitive method of Lemoigne. It was, however, found possible to identify minute amounts of acetoin in a pyruvate medium after growth of *B. subtilis*, an observation which may be associated with Lemoigne's on calcium lactate.

In the case of *B. subtilis*, it is perhaps permissible to speculate on lines suggested by Meyerhof's work: may not the acetoin and its parent acetaldehyde arise from sugar formed by synthesis from the lactate or pyruvate, rather than directly from these substances? Organisms of a strongly aerobic and non-fermenting type, such as *B. subtilis*, may owe these characteristics partly to enhanced synthetic powers; it may be that intermediate products do not accumulate in the medium because they are rapidly resynthesized to carbohydrate as fast as they are formed, a part undergoing oxidation to provide energy for the synthesis, as with muscle. Meyerhof has shown that lactic and pyruvic acids are readily synthesized to carbohydrate both by yeast and by muscle. The distinction between non-fermenting aerobic bacteria (e.g. *B. subtilis* and the Timothy Grass bacillus), and fermenting facultative anaerobes (e.g. *B. coli* and the acetoin-forming soil bacillus) may be in part the same as the distinction between the wild oxidizing yeasts and the cultivated fermenting yeasts (see Meyerhof, 50).

Other Observations on the Production of Acetaldehyde by bacteria.

In view of the results obtained with acetoin, it seemed worth reconsidering experiments made with bacteria in presence of sulphite as a fixative for acetaldehyde. When these were surveyed it was noticeable that, as with acetoin, positive results were always obtained when acetaldehyde was sought in media containing sugar or glycerol, but that with lactate and pyruvate the results were doubtful or negative. Thus Neuberg and Nord (66) isolated acetaldehyde by means of sulphite from fermentations of sugars, mannitol, and glycerol, by *B. coli*, and the bacilli of dysentery and gas gangrene. Peterson and Fred (71) grew three pentose-fermenting bacteria (*B. acetoethylicum, Lactobacillus pentoaceticus*, and an organism of the *colon-aerogenes* group) on media containing pentose or hexose, and obtained acetaldehyde by the use of sulphite; they failed, however, to demonstrate any acetaldehyde in pyruvate media, in spite of good growth. Nagai (53) was able to isolate considerable amounts of acetaldehyde after growth of *B. coli* and *B. lactis aerogenes* on sugar-peptone media in presence of sulphite, and smaller amounts after growth on lactate media. Like so much other work,

Nagai's is made difficult of interpretation by casual addition or absence of peptone.

Both acetoin and sulphite experiments seem to show that acetaldehyde is more freely formed by bacteria from sugars and glycerol than from lactic and pyruvic acids. With the latter may be associated substances such as succinic, malic, and fumaric acids, which from the work of Quastel (72, 73) would seem to be normally metabolized by bacteria *via* oxalacetic and pyruvic acids. That lactic acid belongs to the same group, and is also normally oxidized by bacteria and yeast to pyruvic acid, is clear from the observations of Mazé (48, 49), Quastel, Stephenson and Whetham (75), and others.

The Action of Plants on Pyruvic Acid.

The contrast between the action of bacteria on pyruvic acid and the effect of a yeast fermentation of pyruvic acid is very marked. An experiment described by Neuberg and May (1923) allowed the following balance to be established in such a fermentation. From 13·2 gr. of pyruvic acid were obtained:

4·82 gr. of carbon-dioxide.
2·86 gr. of acetaldehyde (as the dimedon compound).
2·07 gr. of acetoin.
3·36 gr. of unchanged pyruvic acid.

13·11 gr.

Other α-keto acids are attacked in the same way by yeast. The aldehyde resulting from decarboxylation is usually partly recovered as such, and partly as the corresponding alcohol and acid after a Cannizarro reaction between two molecules of aldehyde (compare Neuberg's "third form of fermentation"). Some of the aldehyde may undergo condensation. α-Keto-butyric, α-keto-valeric, α-keto-caproic, and aromatic α-keto acids were used by Neuberg to demonstrate the decarboxylating action of yeast.

The experiments of Nagayama (54) show that moulds such as *Mucor* and *Aspergillus* exhibit towards pyruvic acid a grade of decarboxylative activity similar to that of yeast. He grew six species of moulds on pyruvate media, and isolated 4–24% of the theoretical yield of acetaldehyde; this amount was somewhat increased in presence of sulphite. The resemblance of moulds to yeast is further emphasized by the work of Neuberg and Cohen (59), who cultivated a number of moulds on sugar-containing media in presence of sulphite, and obtained large amounts of acetaldehyde in every case. Many species behaved as yeast does in presence of sulphite, changing sugar to equimolecular quantities of glycerol and acetaldehyde (Neuberg's "second form of fermentation"). Other species with a more aerobic type of metabolism produced no glycerol.

A definite anaerobic production of acetaldehyde and carbon-dioxide from both pyruvate and glucose is also reported for the green plant. For example, Zaleski and Marx (96, 97) showed that powdered peas and beans produced acetaldehyde and carbon-dioxide anaerobically from pyruvate solutions. The same was reported by Bodnár (7) for enzyme preparations of potatoes and sugar beet. Neuberg and Gottschalk (62) made a sterile powder of peas, and suspended it in a glucose solution under anaerobic conditions. After three days they were able to isolate from one experiment 5·5 mg. of acetaldehyde and 3·0 grm. of alcohol, while in a parallel experiment, in presence of sulphite, they found 164·2 mg. of acetaldehyde and only 0·33 grm. of alcohol.

In the plant kingdom, then, the power to decarboxylate pyruvic acid occurs from yeasts and moulds upwards. Acetaldehyde may be produced anaerobically from either pyruvate or glucose, and is a stage in the production of alcohol which is characteristic of the plant under anaerobic conditions. That acetaldehyde is formed also during the normal (aerobic) respiration of the green plant is shown by Klein (33). Yet even with yeast, it is doubtful whether pyruvic acid should be considered an invariable stage between carbohydrate and acetaldehyde. In normal fermentations by yeast juice the intervention of pyruvic acid seems proved by the experiments of v. Grab (24), who used β-naphthylamine as a means of fixation, and obtained about 4% of the sugar fermented as a condensation product of pyruvic acid (α-methyl, β-naphthocinchoninic acid). On the other hand, Hägglund and Augustsson (26) find that at P_H above 7 yeast ferments pyruvic acid very slowly. This they suggest might be explained on the basis of observations by Henri and Fromageot (28) as due to the preponderance of the enol isomeride of pyruvic acid at high P_H, as compared with the preponderance of the apparently more easily decarboxylated keto isomeride at low P_H. If pyruvic acid were formed by yeast during alkaline fermentation, one might thus expect that it would accumulate. Kato (32) and others have found that the accumulation of pyruvic acid in bacterial fermentations is favoured by neutral or alkaline conditions. One is inclined to wonder whether the decarboxylative activity of the plant towards pyruvic acid is not partly due to the more acid reaction of the plant cell, and the consequent shift of equilibrium towards the keto form of pyruvic acid.

The Relations of Acetaldehyde and Pyruvic Acid in the Animal Cell.

With bacteria it is clear that the production of acetaldehyde from pyruvic acid does not occur to anything like the same extent as in the plant. In the animal, also, the power of decarboxylate α-keto acids is never prominent, though the occurrence of acetaldehyde during oxidation of carbohydrate is well established. For example, Hirsch (29) isolated acetaldehyde from chopped frog muscle, using dimedon as a fixative. Neuberg and Gottschalk (60) obtained

acetaldehyde from liver pulp incubated with sulphite, and found an increased yield in presence of glycogen and other substances. The production of acetaldehyde was inhibited by cyanide (Gottschalk, 23). In a later paper, Neuberg and Gottschalk (61) claim that at least 40% to 50% of the carbohydrate oxidized by chopped frog muscle passes through the stage of acetaldehyde. This amount was actually isolated by the use of calcium sulphite, which is by no means a completely efficient fixative. Stepp and Feulgen (88) found small quantities of acetaldehyde in urine. Fabre (16) and Supniewski (91) report acetaldehyde in blood; Gee and Chaikoff (22) confirm its presence in amounts of 2 to 5 mg. per litre.

But turning to pyruvic acid, the experiments of Gottschalk (23) show that liver tissue is capable of changing it into acetaldehyde and carbon-dioxide in very small amounts only. He incubated liver pulp with sulphite, and found that acetaldehyde could be isolated under aerobic conditions in amounts of about 2 mg. per 100 grm. of tissue; in presence of pyruvate (equivalent to 0·2 gr. of pyruvic acid), acetaldehyde was formed anaerobically (2 mg.), and the aerobic yield was increased to 4 mg. Wieland (94), working with a variety of catalysts, found that neither palladium black, charcoal, nor tissues, with the sole exception of unwashed liver tissue, could catalyze the anaerobic decomposition of pyruvic acid into acetaldehyde and carbon-dioxide. On the other hand, he found that all his catalysts, including boiled tissues, could rapidly split off carbon-dioxide from oxalacetic acid:

$$COOH.CH_2.CO\ COOH \longrightarrow CH_3.CO.COOH + CO_2.$$

This point is of interest, since the breakdown of oxalacetic acid is often classed with that of pyruvic acid as indicating the presence of "carboxylase." It would be well to adopt the division into α and β carboxylases made by some writers, and to recognize that oxalacetic acid may be decomposed to pyruvic acid and carbon-dioxide or acetoacetic acid to acetone and carbon-dioxide, by tissues which cannot catalyze the decarboxylative breakdown of pyruvic acid. In other words, carbon-dioxide is more easily split off from β than from α-ketonic acids, a fact which is obvious from the behaviour of β-ketonic acids *in vitro*, and which could no doubt find explanation on the lines of the electronic considerations developed by Quastel (73).

A case illustrating the need for definition is the work of Mayer (46) on the anaerobic breakdown of oxalacetic acid by liver and muscle tissue. He found that when chopped tissue was incubated for 24 hours with oxalacetic acid in buffered solution, about 1 molecule of carbon-dioxide was produced per molecule of oxalacetic acid, as compared with half this amount when oxalacetic acid was incubated alone. The experiments are taken to prove the presence of "carboxylase," but seem rather to suggest that true α-carboxylase is not markedly active under these conditions. A slight breakdown of pyruvic acid probably did occur,

since in some experiments rather more than 1 molecule of carbon-dioxide was produced from 1 molecule of oxalacetic acid, and also since a small amount of oxalacetic acid is not broken down, but is converted into malic acid in presence of tissues (Mayer, 47).

Absence of carboxylase from a liver carcinoma is reported by v. Euler and Myrbäck (15); no measurable amount of carbon-dioxide was developed from a pyruvate solution within 24 hours. These authors are of the opinion that the carboxylase found in animal tissues acts on some other substrate than pyruvic acid, and that the acetaldehyde formation observed by Neuberg and Gottschalk in animals is not connected with carboxylase. Shaffer also, after reviewing the evidence, write (81): "There are difficulties in the acceptance of the view that glucose is oxidized via . . . pyruvic acid and acetaldehyde." Gottschalk, writing in Oppenheimer's *Handbuch der Biochemie* (2nd ed., vol. II) on the breakdown of carbohydrate by animal cells, concludes that facts do not yet permit of a decision as to whether pyruvic acid is the precursor of acetaldehyde in the oxidative breakdown of carbohydrate by muscle.

The Breakdown of Pyruvic Acid by Bacteria.

Before considering an alternative source for acetaldehyde in bacterial and animal metabolism, some evidence as to the non-decarboxylative breakdown of pyruvic acid may be discussed.

Neuberg in 1914 (56) showed that pyruvic acid was broken down to acetic and formic acids by a mixed culture of putrefactive organisms. He explained the products as arising by a hydrolysis of the following type:

$$\begin{array}{l} CH_3.CO \\ | \\ COOH \end{array} + \begin{array}{l} OH \\ | \\ H \end{array} \rightarrow \begin{array}{l} CH_3.COOH \\ + \\ H.COOH \end{array}$$

A similar reaction was found to occur with other α-keto acids: for example, 20% of the theoretical yield of propionic acid was isolated from a fermentation of α-keto-butyric acid, along with some formic acid, carbon-dioxide, and hydrogen (Neuberg, 57). Again, the products of the action of mixed putrefactive organisms on methyl-ethyl-pyruvic acid suggested that the same type of reaction had occurred (Neuberg and Rewald, 68):

$$\begin{array}{l} CH_3 \\ \quad >CH.CO.COOH \\ C_2H_5 \end{array} + HOH \rightarrow \begin{array}{l} CH_3 \\ \quad >CH.COOH \\ C_2H_5 \end{array} + H.COOH$$

It seems worth while to inquire if such a change to the next lower fatty acid + formic acid is not a more usual path for the breakdown of α-keto acids by the animal and bacterial cell than decarboxylation to an aldehyde + carbon-dioxide. It must be recognized that decarboxylation may be followed by oxidation of the aldehyde to acid (or a Cannizarro reaction of 2 molecules of aldehyde

to give alcohol and acid); the products after such a reaction, namely, acid and carbon-dioxide, resemble those of the suggested hydrolysis after oxidation of formic acid has occurred. On the other hand, it is equally possible to suppose that any acetaldehyde produced from pyruvic acid is derived from acetic acid by reduction, though, as we have already seen, acetaldehyde is seldom reported as arising from pyruvic acid.

Thus it is obviously difficult to draw hard and fast lines of distinction. Nevertheless, some evidence is available. Besides Neuberg's work with mixed cultures of bacteria, there are other examples of hydrogen being set free from fermentations of pyruvic acid, and though hydrogen may be readily accounted for as arising from formic acid, it is difficult to see how its origin could be explained on the theory of decarboxylation. Karczag and Móczár (31), using peptone media, found that gas was produced from pyruvate by the same organisms which produce gas from sugar, e.g. *B. coli, B. paratyphoid B.*, and *B. enteriditis* (Gaertner) (not by *B. typhosus, B. dysenteriae, Streptococci,* or *Staphylococci*). The gas was found to contain a large proportion of hydrogen. These authors conclude that "die Brenztraubensäure durch Bakterien eine Veränderung erlitt, die von der Veränderung die diese Substanz durch Hefe erleidet, biologisch und chemisch zu unterscheiden ist." Peterson and Fred (71) found hydrogen produced from pyruvate by an organism of the *colon-aerogenes* group. The present writer has observed the production of hydrogen in considerable quantity from a pyruvate-inorganic medium by the soil bacillus already mentioned. Traces of formic acid can be identified in addition.

On the other hand, many species of bacteria produce no gas when grown on pyruvate-inorganic medium with Durham tubes, including some species which Pakes and Jollyman (70) describe as capable of breaking down formic acid to give hydrogen. And again, there is no accumulation of formic acid in pyruvate media after growth of organisms, which according to Pakes and Jollyman are unable to attack formate. It should be noted, however, that Pakes and Jollyman seem to have employed the production of hydrogen as the only criterion of the breakdown of formate. It is conceivable that some organisms reported by them as not attacking formate did so in reality, transferring hydrogen to acceptors in the medium; of these there was no lack, as 2% peptone was used. Similarly, we may imagine that formic acid produced from pyruvate might be oxidized as soon as it is formed, whether by hydrogen acceptors or by oxygen. Emulsions of *B. coli* and *B. pyocyaneus* will very readily transfer hydrogen from formic acid to methylene blue (see Quastel and Whetham, 77, 78). The concentration of formate in Pakes and Jollyman's experiments is not definitely stated, but seems to have been from 0·5% to 2·0%. It is likely that organisms might give off hydrogen from formate present in such large amounts, but might be able to oxidize it as fast as it was produced from pyruvate. Especially does this seem

likely, since formic acid is most easily pictured as arising from pyruvic in "activated" or "nascent" form:

$$\begin{array}{ccccc} CH_3.CO & & OH & & CH_3.COOH \\ | & + & | & \rightarrow & + \\ C{-}OH & & H & & >C< \begin{matrix} OH \\ OH \end{matrix} \\ O & & & & \end{array}$$

There is a further reason for desiring more information on the subject of formic acid breakdown by bacteria than is available in the work of Pakes and Jollyman: their experiments appear to have been performed under anaerobic conditions, hence the true aerobes are all classed as not attacking formate. Unpublished experiments of Stephenson and Whetham show that at least one aerobe (the Timothy Grass bacillus) can oxidize low concentrations of formic acid when glucose is also present to provide a suitable source of carbon for growth. The whole subject of the breakdown of formic acid by bacteria has remained almost untouched since the time of Pakes and Jollyman, and would repay fresh investigation.

The presence of acetic acid in fermentations of pyruvic acid has often been reported, but, as noted above, this may be explained on the basis of either theory. Acetic and formic acids are among the products of breakdown of succinic acid (E. C. Grey, 25), and fumaric acid (Quastel, 72), both of which substances are probably metabolized by bacteria *via* pyruvic acid (see above).

Observations, probably relevant but not easy to interpret, are those on changes of hydrogen ion concentration in pyruvate media during the growth of bacteria of various species. Some show an immediate increase of alkalinity from an original P_H of 7·4 (*B. pyocyaneus, B. fluorescens*); some grow for a day or two without any change of P_H, and then produce alkalinity (*B. subtilis*); others cause a fall in P_H, which may extend below P_H 6·5, and last for a week or more (*B. coli, B. prodigiosus*, the acetoin-forming soil bacillus). In all cases the final reaction is alkaline, marking the accumulation of base when the pyruvic acid is fully oxidized. The formation of two acids from one by hydrolysis would account for the fall of P_H; it is, however, difficult to demonstrate more than traces of formic acid. The acidity may perhaps rather be attributed to simultaneous removal of organic acid for synthetic processes, accumulation of acetic acid, and utilization of ammonia; where the medium becomes alkaline, one may picture a more rapid removal of acetic acid by oxidation or synthesis. Such points are susceptible of experimental proof. A low P_H during metabolism of pyruvic acid by *B. coli* and other species was recorded by Karczag (30, 31). It is of interest that the P_H changes produced by various bacteria during growth on a pyruvate-ammonia medium are similar to those produced by each species in Cole and Onslow's tryptic broth (casein digest). One may perhaps explain this by recourse to the work of Neubauer (55), and may regard an amino acid mixture as a potential mixture of ammonium salts of α-keto acids.

The energy relationships of the products of pyruvic acid breakdown are worth consideration, especially in view of the fact that anaerobic growth of facultative anaerobes is possible where pyruvic acid is the sole source of carbon (Aubel and Salabartan, 2) (Quastel and Stephenson, 74). The heat of combustion of pyruvic acid, recently determined by Blaschko (5), is identical with that of acetaldehyde; decarboxylation consequently occurs without energy transfer. On the other hand, both the formation of alcohol and acetic acid from acetaldehyde, and the formation of acetic and formic acids from pyruvic acid, are processes involving output of energy:

$$2\ CH_3.CHO + HOH \longrightarrow C_2H_5.OH + CH_3.COOH + 22\ Cal.$$

$$CH_3.CO.COOH + HOH \longrightarrow CH_3.COOH + H.COOH + 6{\cdot}5\ Cal.$$

Either decarboxylation or hydrolytic breakdown could therefore provide energy for synthetic processes during anaerobic growth on pyruvic acid. A third possibility is that suggested by Quastel and Stephenson (74):

$$2\ CH_3.CO.COOH + HOH \longrightarrow CH_3.CHOH.COOH + CH_3COOH + CO_2 + 15\ Cal.$$

Investigation of the products left after anaerobic growth on pyruvic acid might throw light on the processes of breakdown.

The Breakdown of Pyruvic Acid by the Animal Cell.

Passing now to the metabolism of pyruvic acid by the higher animal, it is first to be noted that pyruvic stands in close relationship to lactic acid, and usually undergoes synthetic rather than destructive treatment. Thus in the diabetic or phloridzinised organism, pyruvate gives rise to almost as large an excretion of "extra glucose" as lactic acid (Dakin and Janney, 10). Mayer (45) found lactic acid in the urine of rabbits after administering pyruvic acid, and Embden and Oppenheimer (14) showed an increase of *d*-lactic acid after perfusing surviving dog's liver with blood containing pyruvate. Meyerhof's recent work (Meyerhof, Lohmann and Meier, 51) places pyruvic acid with lactic acid as the only substances which can be re-synthesized to glycogen by muscle.

It is possible that the breakdown of pyruvic acid may be effected by the animal to a small extent under certain conditions. Experiments such as those of Tschernorutsky (92) with liver, and Maeda (44) with placenta, merely show disappearance of pyruvate and production of carbon-dioxide. Embden and Oppenheimer (13) perfused surviving livers with pyruvate, and found an increased production of acetoacetic acid, which they attributed to formation of acetaldehyde, condensation to aldol, and subsequent oxidation:

$$2\ CH_3.CO.COOH \longrightarrow 2\ CH_3.CHO + 2\ CO_2$$

$$2\ CH_3.CHO \longrightarrow CH_3.CHOH.CH_2.CHO$$

$$CH_3.CHOH.CH_2.CHO + O_2 \longrightarrow CH_3.CO.CH_2.COOH + HOH$$

In view of the results of Loeb (39), however, the production of acetoacetic acid from pyruvate might also be explained by direct condensation of acetic acid arising from pyruvate:

$$2\ CH_3.CO.COOH + 2\ HOH \longrightarrow 2\ CH_3.COOH + 2\ H.COOH$$
$$2\ CH_3.COOH \longrightarrow CH_3.CO.CH_2.COOH + HOH$$

Loeb (39) obtained increased acetoacetic acid on adding acetate to the blood perfusing a surviving liver; 3% to 10% of the acetic acid went to acetoacetic, and 30% to 90% disappeared. The formation of acetoacetic acid from acetaldehyde by the surviving liver, observed by Friedmann (21), appears to be less regular and less extensive than the formation from acetic acid. One might even suppose that acetoacetic acid does not come from acetaldehyde *via* aldol, but *via* acetic acid derived from acetaldehyde by a Cannizarro reaction. That liver tissue can catalyze the production of alcohol and acetic acid from acetaldehyde is well established.

In the earlier perfusion experiments no attempt was made to estimate disappearance of pyruvic acid. Simon and Aubel (82) found that only 40% of pyruvate injected into a dog or rabbit could be recovered after 1 hour, but they did not investigate its fate; synthesis rather than breakdown may have been involved. Levene and Meyer (38), using aseptic precautions, incubated kidney pulp and leucocytes with sodium pyruvate; there was no production of carbon-dioxide, and no diminution in pyruvic acid as estimated by the total amount of permanganate required to oxidize substances left after precipitation of protein. These results were confirmed for liver and muscle tissue by Simon and Aubel (83), using the hydrazone of pyruvic acid as a means of estimation.

With the higher animal, as with bacteria, the behaviour of succinic acid indicates that it may be metabolized *via* pyruvic acid. In the diabetic, succinic acid gives rise to glucose; when perfused through a surviving liver, small amounts of acetoacetic acid are formed from it (Ohta, 69). Malonic acid also gives acetoacetic acid in perfusion experiments, no doubt by virtue of a breakdown to acetic acid and carbon-dioxide (Momose, 52).

The general question of the intermediate metabolism of α-keto acids in the body is partly treated by Neubauer (55) in his work on amino acids. His evidence shows that amino acids probably undergo oxidative deamination to the corresponding α-keto acid; also that under certain conditions amino acids give rise to the fatty acid with one carbon atom less. He supposes that the α-keto acids arising from amino acids go by decarboxylation and subsequent oxidation to the fatty acid next lower:

$$R.CHNH_2.COOH + O \longrightarrow \underset{+\ NH_3}{R.CO.COOH} \longrightarrow \underset{+\ CO_2}{R.CHO} + O \longrightarrow R.COOH$$

One may offer the following alternative scheme for the final stages:

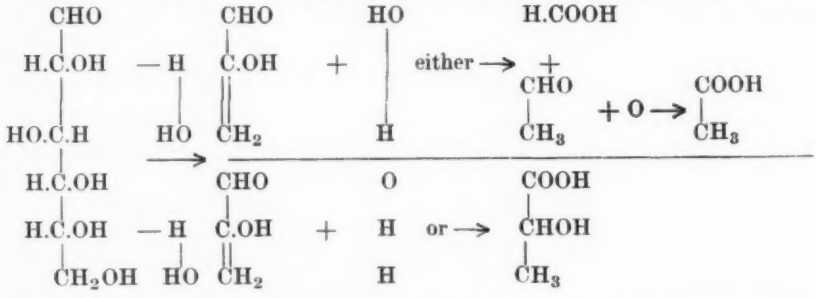
$$R.CHNH_2.COOH + O \longrightarrow \underset{+\ NH_3}{R.CO.COOH} + HOH \longrightarrow R.COOH + H.COOH$$

Formic acid will receive further attention in the next section, but one point may be raised here. On the basis of Wishart's work, Oppenheimer writes in *Die Fermente* (5th ed., p. 1726) that formic acid is inactive as a hydrogen donator to methylene blue in presence of animal tissues, in contrast to the extreme activity found for formates by Quastel and Whetham (77, 78) in presence of emulsions of *B. coli* or *B. pyocyaneus*. Reference to Wishart's paper (95) shows that although formic acid was inactive in presence of muscle extracts, yet in presence of liver extracts it was a better hydrogen donator than any other substance except succinate. Batelli and Stern (4) have shown that animal tissues possess to a marked extent the power of oxidizing formic acid in presence of hydrogen-peroxide. Formic acid administered to the intact animal in moderate doses is readily oxidized. If, then, one postulates a split to fatty acid + formic acid as the first stage in the breakdown of α-keto acids produced in the liver by de-amination of amino acids, there is no difficulty in picturing the immediate destruction of such formic acid.

On the whole, one is tempted to the view that the breakdown of α-keto acids by the animal body and the bacterial cell may follow the hydrolytic path indicated by the researches of Neuberg on bacteria, rather than the decarboxylative path indicated by his discoveries with yeast. If pyruvic acid be broken down to acetic and formic acids, what is to replace it as precursor of the acetaldehyde found during metabolism of carbohydrate?

2. *The Origin and Fate of Acetaldehyde.*

Graham Lusk, in his paper, "Die Energiequelle bei der Muskelarbeit" (1925), formulates the following scheme for carbohydrate breakdown by muscle:



Methyl-glyoxal formed from glucose may be changed (*a*) under asphyxial conditions to lactic acid, or (*b*) under aerobic conditions to acetaldehyde and formic acid. Lactic acid is not oxidized in the body, and is only removed by re-synthesis to carbohydrate. Acetaldehyde is formed during the oxidation of methy-glyoxal, and undergoes further oxidation to acetic acid. It is not produced under

asphyxial conditions; if it were, the body would make alcohol from it, and alcohol is only found in small quantities even after death from asphyxia.

Of direct evidence for this scheme there is none. The question of the oxidation of methyl-glyoxal has been neglected in favour of investigations on the action of glyoxalase in changing methyl-glyoxal to lactic acid. A single fact about phenyl-glyoxal is perhaps pertinent. Dakin and Dudley (9) administered doses of 1·0 to 1·5 gr. of phenyl-glyoxal to rabbits, and besides about 0·5 gr. of mandelic acid, they were able to recover from the urine about 0·75 gr. of hippuric acid, corresponding to 0·5 gr. of benzoic acid. Mandelic and benzoic acids in this experiment may correspond to lactic and acetic acids in the scheme given above.

But a certain amount of indirect evidence is available, and on the whole the scheme appears to accord well with many facts besides those assembled by Lusk. It provides at least a useful alternative hypothesis in place of the pyruvic acid-acetaldehyde theory. As well as explaining the presence of acetaldehyde where carbohydrate is being oxidized, Lusk's scheme also accounts for the equally widespread occurrence of formic acid.

Production and Breakdown of Formic Acid.

This substance has been reported from bacterial fermentations of all kinds. It may be formed (and can be broken down) by yeast growing in aerobic conditions (see Franzen and Steppuhn, 20). Steppuhn and Schelbach (90) found formic acid in the urine of normal dogs and rabbits and in autolyzing tissue; they observed an increased excretion of formic acid by a dog after addition of glucose to the diet, and they claim that formic acid is an intermediate product of carbohydrate breakdown. Dakin, Janney and Wakeman (11) found 80 to 118 mg. of formic acid per day in normal human urine. They give results showing that the daily excretion of formic acid by a dog dropped in starvation to a constant level of 9 mg.; this was increased to 27 mg. on a meat diet and 51 mg. on a potato diet. Formic acid excretion also rose after intravenous injections of glucose. Stepp (87) discovered small amounts of formic acid in normal human blood. It has been shown in the previous section how formic acid might arise in protein metabolism, and Lusk's scheme suggests an origin for it in carbodydrate metabolism; it would appear that small amounts may in each case escape oxidation. Since formic acid in small doses is completely oxidized by the animal body, one may perhaps conclude that the normal excretion represents the breakdown of considerable amounts.

All workers on the subject have recognized that acetaldehyde is, in the animal body, an oxidation product of glucose. On the pyruvic acid theory, oxidation must occur in order that pyruvic acid should be formed from glucose. On Lusk's scheme, it is not at first sight clear why oxidation should be necessary

to produce acetaldehyde, but consideration of the thermal relations illuminates the situation. The reaction glucose $\longrightarrow$ 2 acetaldehyde + 2 formic acid is endothermic: $C_6H_{12}O_6 \longrightarrow 2\ CH_3.CHO + 2\ H.COOH$ — 6·8 Cal. It can thus only proceed if energy is simultaneously obtained from some other reaction. Neglecting acetaldehyde for a moment, one may consider the various possible modes of breakdown of formic acid.

1. With bacteria which liberate hydrogen from formic acid, it would seem that energy for production of acetaldehyde must be obtained from some source other than formic acid, e.g., from the split of carbohydrate to lactic acid. Some bacteria which set free hydrogen during fermentation of sugar, e.g., *B. coli*, change about half the sugar fermented to lactic acid. Aubel (1) interprets the breakdown of sugar by *B. coli* as involving an exothermic split to lactic acid, coupled with an endothermic formation of pyruvic acid and hydrogen; aerobically the hydrogen is oxidized, anaerobically it is set free as such. One may interpret his experimental results equally well on the basis of an exothermic split to lacid acid, coupled with an endothermic production of acetaldehyde and formic acid. The pyruvic acid, he finds (using a medium containing chalk), may arise partly, as he suggests, by oxidation of methyl-glyoxal; another possibility, especially in the aerobic experiments, is oxidation of lactic acid, to which may be due the lower yield of lactic acid aerobically.

2. With other bacteria, and in part with *B. coli*, etc., formic acid is destroyed anaerobically, but hydrogen is not set free. This must involve an oxidation-reduction, from which it may be possible to obtain energy, e.g.:

$CH_3.CHO + H.COOH \longrightarrow C_2H_5.OH + CO_2 + 15$ Cal.

$CH_3.CHOH.CH_2.COOH + H.COOH \longrightarrow CH_3.CH_2.CH_2.COOH + HOH + CO_2 + 27$ Cal.

$COOH.CH = CH.COOH + H.COOH \longrightarrow COOH.CH_2.CH_2.COOH + CO_2 + 27$ Cal.; etc., etc.

Comparing the first equation with that previously given for the breakdown of glucose to acetaldehyde and formic acid, it is apparent that the reduction of acetaldehyde to ethyl alcohol by formic acid yields sufficient energy to provide for a further production of acetaldehyde from glucose—as is, of course, to be expected, since the initial and final stages are those of alcoholic fermentation. It may be that a scheme of this kind actually represents the course of events in alkaline fermentation. It has been suggested above that pyruvic acid may not be an intermediate product in Neuberg's "second" and "third forms of fermentation," and one might assume in these cases a masked formation of formic acid leading to immediate reduction of a $\frac{1}{2}$ sugar molecule to glycerol:

$$C_6H_{12}O_6 \longrightarrow 2\ C_3H_6O_3$$
$$C_3H_6O_3 \longrightarrow CH_3.CHO + H.COOH$$
$$C_3H_6O_3 + H.COOH \longrightarrow C_3H_8O_3 + CO_2$$

3. Finally, formic acid may be oxidized to carbon-dioxide and water, giving ample energy to further the split of glucose to acetaldehyde and formic acid:

$$H.COOH + O \longrightarrow HOH + CO_2 + 63 \text{ Cal.}$$

One must assume this to be somehow the ultimate mode of breakdown in the animal body. Muscle seems unable to catalyze the transfer of hydrogen from formic acid to methylene blue. In the liver, the work of Wishart indicates that some transfer of hydrogen from formic acid to hydrogen acceptors may occur, and it is actually found that production of alcohol under anaerobic conditions—always small in animal tissues—is largest in the liver. It appears as though acetaldehyde were not produced elsewhere except by a reaction coupled with direct oxidation of formic acid.

Production of Acetic Acid.

It is indeed one of the essentials of Lusk's theory that acetaldehyde should only be produced under aerobic conditions, and should undergo immediate oxidation to acetic acid. Acetaldehyde is toxic to the animal body in fairly low concentration, and if large doses are given to an animal there is much disturbance of metabolism; on the other hand, large doses of acetate are easily tolerated and rapidly oxidized. See, e.g. Lusk (42) and Loeb (39) quoted above. The further fate of acetic acid becomes a matter of great interest. Some evidence of its employment in synthetic operations may be discussed, with a view to emphasizing the importance of acetic acid rather than acetaldehyde in such processes.

Acetic Acid in Synthetic Fermentations.

Bakonyi (3), working on the acetone-ethyl alcohol fermentation of starch by *B. macerans* and *B. acetoethylicum*, found that in presence of chalk the proportion of the products was 2 molecules of alcohol to 1 molecule of acetone. Acetaldehyde and acetaldol, $CH_3.CHOH.CH_2.CHO$, were added to fermentations and completely recovered as ⅓ acetone, ⅔ ethyl-alcohol. Added calcium acetate was completely recovered as acetone. Bakonyi supposes that acetaldehyde is produced according to the scheme of Neuberg, hydrogen being set free; 2 molecules of acetaldehyde then undergo condensation to aldol, which yields acetic acid and ethyl-alcohol by hydrolysis; 2 molecules of acetic acid (1 of calcium acetate) give 1 molecule of acetone:

$$2\ CH_3.CHO \longrightarrow CH_3.CHOH.CH_2.CHO$$
$$CH_3.CHOH.CH_2.CHO + HOH \longrightarrow CH_3.CH_2.OH + CH_3.COOH$$
$$(CH_3.COO)\ 2.Ca \longrightarrow CH_3.CO.CH_3 + CaCO_3$$

An alternative and simpler hypothesis is to suppose that acetaldehyde undergoes a Cannizarro reaction to give alcohol and acetic acid, without the intervention

of aldol. Be that as it may, the fact remains that added acetic acid is quantitatively changed to acetone. Acetoacetic acid is an obvious intermediate stage. Here, as with liver perfusions (see above), acetic acid rather than acetaldehyde is indicated as the starting point for synthesis of acetoacetic acid. It may be noted also that acetone is produced from pyruvate by *B. acetoethylicum* (Speakman, 85), yet no acetaldehyde is found when this organism is grown on pyruvate in presence of sulphite (Peterson and Fred, 71).

Similar conclusions as to the importance of acetic acid may be reached from a study of the butyric acid and butyl-alcohol fermentations. Fitz (17, 18, 19) found that butyric acid is produced from glucose, and also from glycerol and lactate, being one of the first to recognize that a synthesis was involved. Acetaldehyde was postulated as an intermediate stage by Buchner and Meisenheimer (8) and Neuberg and Arinstein (58), by means of sulphite, were able to identify acetaldehyde as an intermediate product in butyric fermentations of sugar. From pyruvate they obtained no acetaldehyde, only acetic acid, and no butyric acid was formed. Using a peptone medium containing the decarboxylated anhydride of pyruvic aldol, $CH_3.COH.CO$ / $CH_2.CO$, they found that this compound was changed to butyric acid in large amounts. Consequently they formulate the production of butyric acid and butyl-alcohol from sugar, glycerol, or lactic acid, as occurring *via* pyruvic acid, of which 2 molecules go to pyruvic acid. If this suggestion were correct, it would be surprising that the organisms produce no butyric acid on a pyruvate medium. Might one not rather suppose that pyruvic acid is broken down to acetic and formic acids, and the latter is decomposed to hydrogen and carbon-dioxide so rapidly that no hydrogen remains to reduce such acetoacetic acid as may be formed to butyric acid? It is noteworthy that acetone (and so acetoacetic acid) can be produced from pyruvate by the acetone-forming bacteria. When the butyric acid bacteria are growing on lactic acid, hydrogen would be available from the lactic acid for reduction of acetoacetic acid. The production of butyric acid from "pyruvic aldol" is possible without reduction, by loss of CO_2 and addition of the elements of water, as formulated by Neuberg and Arinstein:

$$\begin{array}{l} CH_3.COH.CO \\ \quad\quad | \quad\quad | \\ \quad\quad CH_2.CO \end{array} \rightarrow \begin{array}{l} CH_3.CH_2 \\ \quad\quad | \\ \quad\quad CH = CO \\ + CO_2 \end{array} + HOH \rightarrow \begin{array}{l} CH_3.CH_2 \\ \quad\quad | \\ \quad\quad CH_2.COOH \end{array}$$

Further information on butyric fermentation is available in the papers of Speakman (84), and Reilly, Hickinbottom, Henley and Thaysen (80). These workers were using organisms which produced from starch butyl-alcohol, butyric acid, acetone, ethyl-alcohol, acetic acid, hydrogen, and carbon-dioxide. Butyl-alcohol and acetone arose late in the fermentation, at the same time as there was

observed a fall in the amounts of butyric and acetic acids. The addition of acetic acid gave a noteworthy increase of acetone (Speakman, 84); propionic and butyric acids gave the corresponding alcohols, and therewith an increased yield of acetone, one may surmise from acetic acid, which would otherwise have been reduced to ethyl-alcohol by the hydrogen which reduced the added fatty acid to alcohol. That the butyric acid arose from acetic acid is suggested by the figures given by Reilly and his collaborators for the ratio of acetic to butyric acid. Acetic acid is present almost alone in the early stages; acetic and butyric are present in the ratio of 1 to 1·25 at the moment when formation of butyl-alcohol and acetone begins; after this there is a fall in the relative amount of butyric. These authors report the recovery as acetone of at least 72% of added acetic acid. They postulate the formation of acetone from acetic acid via aceto-acetic acid, and they found that addition of acetoacetic ester to a fermentation led to an increased yield of acetone. They do not suggest that acetoacetic acid may be reduced to butyric acid, but this appears a likely sequel. The occurrence of higher fatty acids in a butyric fermentation has been recorded by Neuberg and Arinstein (58). The marked effect of acetic acid in increasing the fat content of the Timothy Grass bacillus may also be recalled (86). Is it by a path of simple condensation that acetic acid is built up into the long chains of the higher fatty acids?

$$CH_3.COOH + CH_3.COOH \longrightarrow CH_3.CO.CH_2.COOH + CH_3.COOH$$
$$\longrightarrow CH_3.CO.CH_2.CO.CH_2.COOH, \text{ etc.}$$

On the views of Quastel (73), the first effect on a saturated fatty acid chain of an activating mechanism, such as is found in the living cell, is the production of an activated molecule of unsaturated type:

$$\underset{\text{Normal}}{-CH_2-CH_2} \longrightarrow \underset{\text{Activated}}{-\overset{\vee}{C}-CH_2-} + 2H$$

The product of oxidation of the activated form may be $-CH=CH-$, $-CHOH-CH_2$, or $-CO-CH_2-$ (Quastel, 73, p. 185. These groups may be formed simultaneously and exist in equilibrium. Leathes has shown that extensive desaturation of fatty acid chains occurs in the liver. On Quastel's view, such unsaturated linkages would occur in the β-position to the carboxyl group, and might exist in equilibrium with keto groups. Simple hydrolysis at α-keto group to give acetic acid is usually assumed to occur during β-oxidation of fats. Wakeman and Dakin (93), working on the decomposition of β-oxybutyric and acetoacetic acids by liver enzymes, obtained incomplete evidence pointing to an increase of acetic acid after disappearance of acetoacetic acid.

In synthesis, one might imagine the converse reactions, namely, establishment of equilibrium between keto groups arising by condensation of acetic acid, and the corresponding unsaturated groupings; reduction of unsaturated to

saturated compounds, and removal of saturated compounds by synthesis into neutral fats. The necessary material for formation of fat would be accumulation of acetic acid, and available hydrogen (e.g. formic acid). Both these are at hand where rapid breakdown of carbohydrate is occurring, and it seems that the necessary transfer of hydrogen can be effected by the liver. Of interest in this connection are the experiments of Lublin (41), showing that R.Q.'s above 1·0 are consistently found after administration of insulin and sugar to the normal man, i.e., rapid oxidation of carbohydrate, rather than presence of excess, favours conversion into fat.

The Oxidation of Acetic Acid.

One further point may be quoted from Lusk's paper. He speaks of the possibility that "micro-explosions" of acetic acid arising from fat by β-oxidation may be responsible for the energy required in the recovery process of muscle. On his view, under normal conditions both methyl-glyoxal and fat may be oxidized *via* acetic acid to provide this energy; in starvation or diabetic conditions fat may be the sole fuel. It is, of course, obvious, though perhaps still worth mentioning, that the R.Q. of acetic acid is unity. The discrepancy between the R.Q. of muscular work and the total R.Q. of the organism during carbohydrate inanition could be explained by assuming local combustion of acetic acid, after desaturation and β-oxidation of fats in the liver. Liver R.Q.'s of the necessary low values have been recorded by Loeb (40); the R.Q. of a fatty liver from a phloridzinized animal was as low as 0·2. On this view the necessity of assuming any extensive formation of carbohydrate from fat would be removed, and, though this process may occur, the evidence indicates that it must be at best a very slow and difficult one.

The conclusions of Raper and Smith (79) are noteworthy. They are led by their work on insulin and the production of acetone bodies by the perfused liver to the following position: "In the presence of adequate supplies of glycogen, only a minimal amount of fat is utilized for supplying the body's needs, and the acetone bodies produced from this small amount of fat are readily oxidized. When, however, the glycogen content of the liver is reduced to a low value . . . fat is called up from the depots, and, accumulating in the liver, is oxidized more abundantly. Over-production of acetone bodies results. . . . In this sense, it may be said that the liver has two types of metabolism at its disposal, which may be termed the carbohydrate type and the fat type, depending on the preponderance of one or the other reserve substance." Or, in other words, the liver has two sources of acetic acid, which comes to appear as the main fuel of the body. One is inclined to allot to it the place others have assigned to pyruvic acid—that of central meeting point of the metabolism of proteins, carbohydrates, and fats. From fats and some amino acids it arises by β-oxidation of

the fatty chain, from carbohydrates by oxidation of acetaldehyde, and from the sugar-forming amino-acids, either via sugar or possibly by breakdown of pyruvic acid. From acetic acid fat synthesis begins, and probably glycine formation; that it may be rebuilt into carbohydrate seems probable for yeast (Meyerhof, 50), and for bacteria which can use acetic acid as sole source of carbon, but for the animal body this is not proven.

Nothing is known as to the mode of oxidation of acetic acid by the living cell, a matter that now becomes of the keenest interest. Possible intermediate substance, such as glycollic and glyoxylic acids, are less readily oxidized than acetic acid itself. Reilly and his collaborators (80) have speculated on the existence of acetic acid in the reactive form $CH_2 = CO$. In presence of bacteria and muscle tissue, acetic acid acts towards methylene blue as a hydrogen donator of low intensity (Wishart, 95; Quastel and Whetham, 77, 78). It may conceivably undergo synthesis to succinic acid in some degree; succinic acid arising during fermentations of sugar by *B. coli* is usually supposed to originate in this manner. Oppenheimer (*Die Fermente*) suggests that acetic acid may be metabolized via succinic, oxalacetic, and pyruvic acids, back to acetaldehyde. It would seem that the oxidation of acetic acid to carbon-dioxide and water must represent two-thirds or more of the energy supply of the animal body. Anything which might help us to discern the processes involved would illuminate a dark and difficult area at the heart of the subject of biological oxidations.

SUMMARY.

1. The relationship of pyruvic acid and acetaldehyde in bacterial and animal metabolism is considered. Enquiry is made as to the way in which pyruvic acid is broken down by bacteria and by the animal body.

2. The origin of acetaldehyde during the oxidation of carbohydrate is discussed on the basis of a suggestion made by Lusk; reference is also made to the subsequent fate of acetaldehyde.

I am glad to have this opportunity of offering my warmest thanks to Professor Sir F. G. Hopkins, Miss M. Stephenson, and Mr. A. B. Anderson for much help and advice. My thanks are also due to the Medical Research Council for a grant.

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ACHROMOBACTER PEROLENS *(n.sp.)* THE CAUSE OF "MUSTINESS" IN EGGS

by

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(Submitted for publication 26th November, 1926.)

Our attention was drawn to the problem of the cause of "mustiness" in eggs by Messrs. Rugg and Clinton, of the Produce Division, Department of Agriculture. Musty eggs have a very characteristic odour, which cannot be masked by pastrycooks in the ordinary manufacturing processes. The presence of such eggs in a mixture may, therefore, lead to the loss of much valuable food.

An egg was received on June 11, 1926. It had come from a cool store, and was stated to be a typical "musty" egg. It possessed a peculiar characteristic smell, which was very penetrating, and persisted in the cupboard, in which the egg had been placed overnight, for several days. The egg was sterilized externally with alcohol, and was broken carefully into a sterile Petri dish. A very strong odour was observed. The yolk appeared quite normal and the yolk membrane strong, but the white of the egg showed a definite opalescence suggestive of bacterial contamination. Examination of stained smears made direct from the egg-white showed numerous small gram negative, somewhat bipolar cocco-bacilli, apparently present in a pure state. Slopes of nutrient agar and other ordinary culture media were inoculated with the egg-white and placed in the incubator at 37°C. No growth was apparent after several days. The tubes of inoculated media were then removed to a cupboard at room temperature, and after twenty-four hours very small, round, semi-translucent colonies became apparent. They increased in size in the next twenty-four hours, and a strong musty odour was observed. The micro-organism was apparently present in pure culture, but single colonies were selected and replated to ensure purity. The following are the characteristics that have been observed.

(*a*) Morphology and Staining Reactions. The micro-organism is small, and varies in shape from that of an imperfect sphere to that of a cocco-bacillus, with occasional longer, round-ended, bacillary forms showing in the cultures. It does not readily form chains, but a few short chains may be met with in broth cultures. Its width is fairly constant. The limits of size in broth are from 0·4μ

to 2·55μ long by 0·35μ wide, when stained with toluidine blue. On agar its size tends to remain more constant, and varies from 1·2μ to 1·4μ long by 0·3μ wide. It is aerobic, not growing in media under a vaseline seal; does not form spores; is gram negative, non-acid fast, and stains well with the ordinary anilin dyes, though the bulk of its staining material is concentrated at each end, so that stained specimens show a bipolar appearance. It is quite motile, and when stained by the simple and excellent method of T. Inouye, is seen to possess a single polar flagellum, usually with two undulations. When thus stained, the average size of organism from agar slopes is 1·6μ long by 0·7μ wide, while the flagellum is from 5μ to 6μ long.

(*b*) Physiology. The temperature limits have not been ascertained, but it has been determined that the organism will not grow at 37°C, which suggests that it cannot be an inhabitant of the body of the fowl; but it grows well at room temperatures, and grows fairly well, though slowly, in the ice-chest. On all media it soon produces its characteristic odour, though the intensity of the smell varies in degree on different media. Sown into sugar-free litmus, Lemco broth, and Durham tubes, with the addition of 1% of the various carbohydrates and alcohols, the following fermentation reactions were obtained after 14 days at room temperature:

(i) Acid but no gas is produced in glucose, levulose, galactose, glycerin, mannit, arabinose.

(ii) No change is produced in saccharose, maltose, lactose, raffinose, dulcit, salacin, inulin.

It does not produce indol (Ehrlich's test). Nitrates are reduced within 24 hours to nitrites, which quickly become reduced to ammonia. Litmus is slowly reduced.

(*c*) Cultural characteristics. After 72 hours at room temperature:

Agar Stroke	Moderate growth; whitish by reflected and semi-translucent to transmitted light. The growth is glistening, somewhat raised along the course of the stroke, but spreads as a transparent flat growth of about $\frac{1}{8}$ in. to $\frac{1}{4}$ in. from the stroke, with finely undulant border. It has a butyrous consistency, and the medium is not coloured. Typical odour very strong.
Broth	A good turbid growth, with slight pellicle and a flocculent sediment. Typical odour very strong.
Glycerine Agar	As on agar, but growth is slightly less abundant; odour strong.
Martin's Agar with 8% Horse Serum	Abundant, filiform, convex, glistening, smooth, semi-opaque, butyrous growth, with no pigment in the medium.
Inspissated Serum	Liquefaction along the line of growth, with a pale, lemon fluorescent tint of the growth.

Dorset's Egg	Abundant, spreading, raised, glistening, smooth growth, the accumulated growth in the water of condensation having a slight orange colour.
Agar Stab	Growth occurs best at the top, with the line of puncture beaded.
Gelatine Stab	Growth occurs best at the top. The line of puncture is beaded, and crateriform liquefaction occurs. The medium is not coloured.
Litmus Milk	No apparent change.

In Martin's broth with serum the growth is similar to that in ordinary broth, but is heavier when horse serum is used instead of ox serum. No growth takes place under anaerobic conditions, such as in liver broth under vaseline seal. Cooked meat medium shows no change of colour.

Further incubation up to three weeks at room temperature causes some changes in the appearance of the cultures. The agar stroke shows a wider film of growth. The broth becomes fairly clear, with a heavy deposit and a thick pellicle. In Martin's broth with 8% ox serum a faint lemon-yellow fluorescence develops. There is complete digestion of the slope of inspissated serum. There is no increase of growth along the line of inoculation in the agar stab, but the growth spreads over the surface as a glistening, circular disc with a thicker, cribiform centre. The growth on glycerine agar is heavier than on plain agar, and the centre raised track is finely wrinkled. The gelatine stab shows an even liquefaction to a depth of 2 cm., with a heavy deposit in the liquefied gelatine. There is very little growth along the track below this level. On potato there is a fairly thick, glistening, raised growth with a brownish tinge. Litmus milk turns red, with gradual decolourization of the litmus and a partial, fine clotting of the caseinogen.

Transmission Experiments.

Fresh, normal eggs were used in the experiments. They were divided into two groups, one being washed and the other being unwashed. The treated eggs were washed well with sand-soap and warm water in an attempt to remove the "bloom," or natural fatty or waxy covering of the egg-shell, which is said to be protective in function.

Two eggs from each group were treated by rubbing a broth culture of the micro-organism over a small area of the shell in each case. They were then placed in a Mason fruit-jar, the atmosphere of which was kept moist by the presence in the jar of moistened cotton wool. The eggs were removed from the jar after six days and examined. They were sterilized externally and broken into sterile Petri dishes. The eggs that had been washed showed distinct opalescence of the white and a more marked odour than the unwashed eggs, the whites of which

were not distinctly opalescent. Cultural examination showed a pure growth of the micro-organism, with typical odour, but there were fewer colonies developing from the white of the untreated than from the washed eggs.

In another experiment two eggs from each group were placed in a jar of broth inoculated with the organism, and were examined after six days, with results similar to those obtained in the previous experiment. It thus appears that this flagellated organism can penetrate through the shell of the egg and infect the contents.

Since the first musty egg was examined another has been received from a different source. From this an organism, apparently similar to, if not identical with, that obtained in the first case, has been isolated.

Under the classification adopted by the American Society of Bacteriologists the organism responsible for "mustiness" in eggs belongs to Family 4—Bacteriaceae; Tribe 2—Achromobactereae; Genus 5—Achromobacter, which are characterized as non-pigment-forming (at most no pigment formed on agar or gelatin) rods occurring in water and soil; motile or non-motile; gram negative. The type species is *Achromobacter liquefaciens* (Frankland).

The organism now described most closely resembles in general characters *Achromobacter liquidum*, but this latter organism does not change litmus milk, and has a thick, flesh-coloured, rough, moist growth on potato. In addition the "musty egg" organism is quite characteristic in the odour which it produces. On account of this characteristic and penetrating, musty odour, the specific name *perolens* (L. perolere, to emit a penetrating odour), appears appropriate for the organism. The name proposed for the organism is, therefore, *Achromobacter perolens*.

SUMMARY.

1. Mustiness in eggs is caused by the infection of the eggs with a specific organism, *Achromobacter perolens* (n. sp.).

2. The possibility of eggs becoming infected in the body of the fowl is remote, since the organism will not grow at the temperature of the fowl's body.

3. Since the organism grows at room temperature it is probable that infection takes place after the egg is laid.

4. As the organism will grow slowly at or slightly above 0°C., it is possible that it may infect eggs in the cool stores.

Experiments to determine this are indicated.